OVERCOMPENSATION OF IPOMOPSIS AGGREGATA FOLLOWING UNGULATE HERBIVORY: GETTING TO THE ROOT OF IT

BY

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DISSERTATION

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ABSTRACT

The idea that some plants benefit from being eaten is counterintuitive, yet there is now considerable evidence demonstrating enhanced fitness following herbivory, i.e., plants overcompensate. The mechanistic underpinnings of overcompensation, however, are unclear. For example, it has been assumed that plants growing in high resource conditions are best able to compensate for herbivory. However, just the opposite has been found for dicotyledonous plants exhibiting patterns of overcompensation; with most occurring in resource-poor conditions. These studies, however, have ignored the potential effects of below-ground interactions with other organisms such as arbuscular mycorrhizal fungi (AMF) and other fungal associates that could provide the necessary nutrients needed.

A long-term study of the monocarpic biennial, scarlet gilia, *Ipomopsis aggregata*, showed that ungulate herbivory by mule deer and elk can result in a three-fold increase in plant fitness. We hypothesized that fungal associations would facilitate the compensatory response most commonly observed in an Arizona population of scarlet gilia; perhaps mutualistic associations with fungi, such as arbuscular mycorrhizal fungi, would explain the phenomenon of overcompensation altogether. However, fungal removal experiments indicate that fungi are not mutualistic but parasitic. The reduction in fungi altered the compensatory response, particularly under water limited conditions, increasing compensation from equal to over. In a year of normal precipitation, a fungicide treatment reduced the compensatory response from overcompensation to a marginally significant trend toward overcompensation, by simultaneously increasing the reproductive success of both browsed and unbrowsed plants. We also show experimentally that the interactive effects of water and fungicide maximize fruit production following ungulate herbivory. Overall, our results are counter to the "modification of tolerance hypothesis", that

plants associating with mycorrhizal fungi will have higher tolerance to herbivory. It is likely that arbuscular mycorrhizal fungi and dark septate endophytes are competing with plants for nutrients, carbon and water following herbivory, limiting the magnitude of compensation.

Both water availability and herbivory also had significant effects on levels of fungal colonization and species distribution patterns. Specifically, drought conditions enhanced overall levels of fungal colonization. In addition, ungulate herbivory, lead to higher colonization of hyphae and arbuscules under drought conditions. It is likely that under drought conditions, fungi colonize browsed plants at higher levels due to the root becoming a sink for carbon, generated by regrowth tissue following the release of apical dominance. Ungulate herbivory also enhanced, or tended to enhance, the richness and diversity of the fungal community in roots and soils. An increase in species following browsing, particularly *Scuttellospora* sp. may indicate that specific fungal species are responsible for the parasitic response of the soil fungal community on the compensatory response of *I. aggregata*. These results suggest that soil fungal community loads and fungal species dictate the magnitude of fitness compensation following ungulate herbivory.

These studies on the interactive effects of herbivory and soil fungal communities on the compensatory response of *I. aggregata* were extended by incorporating a series of phosphorus (P) treatments (the primary nutrient supplied to a host plant by mycorrhizal fungi) to assess the interactive effects of P on plant compensation and arbuscular mycorrhizal colonization. Results show that soil nutrient levels and the soil inhabiting fungal community interact with herbivory to determine the growth and compensatory response of scarlet gilia. Specifically, the compensatory response is limited by P availability, with nutrient availability being more important for browsed plants than for unbrowsed plants. Phosphorus is also shown to decrease mycorrhizal allocation to hyphae, arbuscules and internal spores within plant roots. Whereas plants equally compensated

under low nutrient conditions, they overcompensated under the highest level of P, due to an increase in the fitness of browsed plants. Furthermore, the removal of soil fungi with a fungicide treatment resulted in overcompensation under the lowest and highest nutrient conditions, due primarily, to an increase in the fitness of browsed plants. These results support the findings of others that under high P conditions plants shift to soil P when plants are able to obtain nutrients through their own root systems, decreasing fungal colonization and enhancing plant fitness following herbivory.

This study represents one of few to consider the interactive effects of mammalian herbivory and fungal associations on plant fitness and represents the only study addressing the phenomenon of overcompensation. It also represents one of few studies focusing on plant reproduction as opposed to biomass alone; aboveground biomass and fruit production were at best weakly correlated (R^2=0.29, p=0.001), thus biomass may not give a clear picture of changes that are ultimately of evolutionary importance.

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CHAPTER 1: GENERAL INTRODUCTION

Plant tissue loss to herbivores is an important selective agent shaping plant phenotypes. To date, most studies of plant adaptation have focused on the evolution of defensive traits that reduce or prevent tissue damage by herbivores (Berenbaum et al. 1986, Mauricio and Rausher 1997, Agrawal 1998). However, herbivores may also evolutionarily select for traits that allow plants to maintain fitness in the face of tissue loss (Stowe et al. 2000). Plant genotypes that can compensate for tissues lost with little or no decrement in fitness relative to those that are undamaged represent such an example and are termed tolerant (see Stowe et al. 2000 for a recent review).

Ecologists and evolutionary biologists became interested in tolerance in the mid-1970's when several authors (Chew 1974, Harris 1974, Dyer 1975, Owen and Wiegert 1976) reported that herbivory may result in an increase rather than a decrease in the growth and reproductive success of some plant species (Whitham et al. 1991). This observation was initially dismissed as the result of reallocation of belowground resources to aboveground structures in perennial plants, eventually resulting in a net fitness decrement (Belsky 1986, Verkarr 1986). Studies by Paige and Whitham (1987) provided the first convincing evidence that herbivory can, under some circumstances, lead to enhanced plant fitness (termed overcompensation). Their choice of a monocarpic plant (i.e., one that reproduces only once and then dies) simplified the estimation of lifetime fitness and eliminated the possibility that apparent overcompensation came at the expense of future reproduction (Vail 1992). They showed that when ungulate herbivores remove 95% or more of the aboveground biomass of the monocarpic biennial scarlet gilia, *Ipomopsis aggregata*, the product of lifetime seed production, seed germination, and seedling survival averaged three times that of the uneaten controls (Paige and Whitham 1987, Paige 1992a,b, 1994, 1999, Anderson and Paige 2003). The increase in relative fitness was largely because of an architectural change in the plant; ungulate removal of scarlet gilia's single inflorescence resulted

in the production of multiple flowering stalks due to the release of apical dominance and an overall increase in both above- and below-ground biomass. Although the majority of studies that have considered the effects of herbivory on plant reproductive success have been limited to the seed-bearing success achieved through maternal function (Quesada et al. 1995), more recent studies have also demonstrated that ungulate herbivory on *I. aggregata* results in an increase in paternal as well as maternal fitness (Gronemeyer et al. 1997). Thus, the apparently paradoxical phenomenon of overcompensation could no longer be summarily dismissed (Stowe et al. 2000).

With an increasing number of investigators seeking evidence for overcompensation, more supportive evidence is being uncovered. For example, evidence for increased flower, fruit, and seed production following herbivory has been found for a number of plant species including two species of *Ipomopsis*, *I. aggregata* and *I. arizonica* (Paige and Whitham 1987, Maschinski and Whitham 1989), and several unrelated to *Ipomopsis* including, *Gentianella campestris*, *G. amarella* (Nilsson et al. 1996, Lennartsson et al. 1997), *Sanicula arctopoides* (Lowenberg 1994), *Bouteloua gracilis*, *B. hirsute* (Alward and Joern 1993), *Ipomoea purpurea* (Hougen-Eitzman and Rausher 1994), *Arabidopsis thaliana* (Mauricio et al. 1997, Weinig et al 2003) and *Erysimum strictum* (Rautio et al. 2005). There are also many plants that produce more flowers following herbivory that do not overcompensate in terms of fruit and seed production, but may overcompensate when the paternal contribution to fitness is considered, such that total lifetime fitness is enhanced (Paige et al. 2001).

A relatively large number of studies have been conducted on the compensatory responses of *I. aggregata* ranging from the effects of multiple herbivores and their interactions on male and female components of plant fitness (Paige and Whitham 1987, Paige 1992a, Gronemeyer et al. 1997, Juenger and Bergelson 1998, Anderson and Paige 2003, Juenger et al. 2005) to alternative selection pressures in the removal of apical dominance (Paige 1992b), the interactive effects of the timing of herbivory,

nutrient availability and plant competition (Maschinski and Whitham 1989, Paige 1992a), the direct and indirect effects of drought (Levine and Paige 2004), inbreeding depression and stress effects (Heschel and Paige 1995), effects on pollinators and pollination (Gronemeyer et al. 1997, Juenger and Bergelson 1997, 1999, Paige et al. 2001), geographic patterns of herbivory (Bergelson and Crawley 1992 a,b, Paige 1994, Paige 1999), and the quantitative genetics of tolerance (Juenger and Bergelson 2000). In spite of all we know about fitness compensation in *I. aggregata*, no one has studied the effects of mycorrhizal fungi on compensation in *Ipomopsis* or, for that matter, any other plant species showing patterns of overcompensation following herbivory. In fact, to date, relatively few studies have focused on the interactive effects of herbivory, plant fitness (as opposed to just biomass), and mycorrhizal fungi (Borowicz 1997, Gehring and Whitham 2002, Bennett et al. 2006).

Mycorrhizal fungi form symbiotic associations with approximately 80% of all plant species; associations that can often be mutually beneficial to both plant and fungus (Smith and Read 2008). Associations with mycorrhizal fungi can increase plant access to scarce or immobile soil minerals, particularly phosphorus and nitrogen, and provide other services such as disease/pathogen protection and improved water relations (Harley 1989, Bethlenfalvay 1990, Allen 1991, Sharma and Mukerji 1992, Watkins et al. 1996, Borowicz 2001, Hodge et al. 2001, Whitfield 2007). In turn, mycorrhizal fungi receive up to 4-20% of a plants' photosynthate production (Smith and Read 2008).

There is considerable evidence that plant-mycorrhizal interactions are not always mutualistic but span a continuum from mutualistic to parasitic (Johnson et al. 1997, Bennett et al. 2006). The outcome of these interactions may be contingent upon developmental or environmental factors. For example, parasitic mycorrhizal associations might occur at specific developmental stages such as early seedling growth when mycorrhizal fungi could decrease nutrient allocation or allocation to defense leading to a decrease in seedling survival. Altered mycorrhizal associations may also occur when the

biotic, chemical, or physical environment causes net costs to exceed net benefits (e.g., when plant fitness is reduced rather than enhanced in the presence of mycorrhizal fungi) (Johnson et al. 1997, Klironomos 2003). For example, in environments high in soil nutrients, mycorrhizal fungi may parasitize plants (Johnson et al. 1997, Bennett et al. 2006), given that mycorrhizas are no longer supplying the nutritional needs of the plant.

An association with mycorrhizal fungi may also change the outcome of biotic interactions between plants, their parasites, pathogens and herbivores (Johnson et al. 1997, Borowicz 2001, Gange and Brown 2002, Gehring and Whitham 2002, Bennett et al. 2006). For example, mycorrhizal fungi have been shown to interfere with plant pathogen attack (reviewed in Borowicz 2001). Gange and West (1994) showed that fungal colonization reduced herbivore damage by a leaf chewing lepidopteran, *Arctia caja*, on *Plantago lanceolata*. Gehring et al. (1997) have shown a negative effect of scale insect herbivory on ectomycorrhizal colonization. These seemingly contrasting results suggest that mycorrhizal fungi may compete with herbivores for limiting photosynthates (Borowicz 1997).

In addition, plant growth responses can range from parasitic to mutualistic depending upon the particular species mycorrhizal fungi that are associated with the plant (Klironomos 2004). A rstudy by Klironomos (2004) showed high variability in plant response to the presence of species of mycorrhizal fungi, ranging from parasitic to mutualistic. This seemed to be the rule rather than the exception – each of ten plant species crossed with ten different species of mycorrhizal fungi (crossed one at a time) showed variation, ranging from parasitic to mutualistic in terms of plant biomass, when exposed to individual mycorrhizal species in their home site. Bennett and Bever (2007) found that individual AMF species varied in the effect on tolerance of *Plantago* to natural herbivory, from positive to negative, but, when combined, one species exerted disproportionately positive effects. By

contrast, Gange (2001) found that colonization by a single species of AMF increased resistance of domestic strawberry to root weevils, but this effect disappeared when two AMF species were combined. Clearly the outcomes of such interactions are complex; if there are general trends in the effects of mycorrhizal fungi on plant-herbivore/plant-pathogen interactions, it may not be apparent until more fungus-plant-herbivore-pathogen systems are examined over a range of environmental conditions (Borowicz 2001).

The benefits of mycorrhizal fungi tend to be greatest when soil phosphorous levels are at or below 50 mg kg^{-1} (Schubert and Hayman 1986). Mycorrhizal infection of roots declines above this level, with little if any infection occurring above 100 mg kg^{-1} phosphorous, even when soil is inoculated with a mix of mycorrhizae (Schubert and Hayman 1986), although this may not be the case for all plant species. It has been noted that mildly nutrient-stressed plants tend to release more soluble carbohydrate in root exudates making better AMF hosts than unstressed plants (Sylvia and Neal 1990, Schwab et al. 1991, Johnson 1993). Higher phosphorous concentrations may also select for strains of AMF that are inferior mutualists, acquiring carbohydrates that the host plant has not allocated to it (Johnson 1993). Thus, fertilization may result in less carbohydrate allocation to root exudates, selecting for more parasitic AMF. If these patterns are generally true, we would expect to see variation in scarlet gilia's compensatory response to different species of mycorrhizal fungi, as well.

This dissertation addresses the interactive effects of ungulate herbivory, soil fungi, and environmental conditions on the compensatory response of *Ipomopsis aggregata* by addressing the following questions:

1. A. What is the effect of belowground fungal community on the compensatory outcome of Ipomopsis aggregata following ungulate herbivory? A'. Do they facilitate or inhibit the

compensatory response (i.e., are they mutualistic or parasitic) and B. Are the interactions among ungulate herbivory, soil root fungi and plant compensation altered under varying precipitation?

2. How do the combinatorial effects of herbivory and drought on *Ipomopsis aggregata* affect AMF allocation (hyphae, arbuscules, vesicles and spores) and/or the AMF taxa involved, and, in turn, the compensatory response?

3. A. Is the compensatory response of *I. aggregata* limited by P in Arizona soil? Is there a differential effect of P on unbrowsed versus browsed individuals? B. Do increasing levels of P inhibit or enhance AMF colonization? C. Do increasing levels of P inhibit or enhance the parasitic effects of AMF on fitness compensation of *I. aggregata*?

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CHAPTER 2: BELOWGROUND FUNGAL ASSOCIATIONS AND WATER INFLUENCE THE COMPENSATORY RESPONSE OF *IPOMOPSIS AGGREGATA*

Abstract

A long-term study of the monocarpic biennial, scarlet gilia, *Ipomopsis aggregata*, showed that ungulate herbivory by mule deer and elk could result in a three-fold increase in plant fitness. Here we hypothesized that fungal associations would facilitate the compensatory response most commonly observed in this Arizona population of scarlet gilia; perhaps mutualistic associations with fungi, such as arbuscular mycorrhizal fungi, would explain the phenomenon of overcompensation altogether. However, fungal removal experiments indicate that fungi are not mutualistic but parasitic. The reduction in fungi altered the compensatory response, particularly under water limited conditions, increasing compensation from equal to over. In a year of normal precipitation, a fungicide treatment reduced the compensatory response from overcompensation to a marginally significant trend toward overcompensation, by simultaneously increasing the reproductive success of both browsed and unbrowsed plants. We also show experimentally that the interactive effects of water and fungicide maximize fruit production following ungulate herbivory. Overall, our results are counter to the "modification of tolerance hypothesis", that plants associating with mycorrhizal fungi will have higher tolerance to herbivory. It is likely that arbuscular mycorrhizal fungi and dark septate endophytes are competing with plants for nutrients, carbon and water following herbivory, limiting the magnitude of compensation.

Introduction

The idea that some plants benefit from being eaten is counterintuitive, yet there is now considerable evidence demonstrating enhanced fitness following herbivory, i.e., plants overcompensate (e.g., see Paige and Whitham 1987; Maschiniski and Whitham 1989; Alward and Joern 1993; Lowenberg 1994; Nilsson *et al*. 1996; Lennartsson *et al*. 1997; Mauricio *et al*.

1997; Juenger *et al*. 2000; Weinig *et al*. 2003; Rautio *et al*. 2005; Scholes and Paige 2011, among others). The mechanistic underpinnings of overcompensation, however, are unclear (but see Scholes and Paige 2011, Siddappaji et al. 2013, Scholes and Paige 2014). For example, it has been assumed that plants growing in high resource conditions are best able to compensate for herbivory (Bryant et al. 1983, Coley et al. 1985, Maschinski and Whitham 1989). However, the opposite was found for dicotyledonous plants exhibiting patterns of overcompensation; most occurred in resource-poor conditions (Hawkes and Sullivan 2001, Wise and Abrahamson 2007). These studies, however, have ignored the potential effects of below-ground interactions with other organisms, such as arbuscular mycorrhizal fungi (AMF) and other fungal associates that could provide the nutrients needed.

Arbuscular mycorrhizal fungi form symbiotic associations with approximately 80% of all plant species; associations that can be mutually beneficial to both plant and fungus (Smith and Read 2008). Association with mycorrhizal fungi can increase plant access to scarce or immobile soil minerals, providing up to 70% of phosphorus and 30% of nitrogen uptake, and provide other services such as improved water relations (Smith and Read). In turn, mycorrhizal fungi receive 4-20% of a plants' photosynthate production (Jakobsen et al. 2002, Smith and Read 2008). Another important and less studied group of root fungi are the dark septate endophytes (DSE), a miscellaneous group of ascomycetes that can form beneficial relationships with the host plant much the same as AMF (Jumpponen 2001). In addition, saprophytic fungi that degrade non-living organic matter have the potential to increase availability of mineralized nitrogen and phosphorus for plant uptake. An association with non-pathogenic fungi that delivers nutrients could contribute to resource conditions needed for an overcompensation response.

Here we are interested in the role of fungal associations on the compensatory response of monocarpic biennial scarlet gilia, *Ipomopsis aggregata*, following ungulate herbivory. In spite of all we know about overcompensation in scarlet gilia, no one has studied the effects of fungal species on plants showing patterns of overcompensation following herbivory. In fact, to date, relatively few studies have focused on the interactive effects of herbivory, plant fitness (as opposed to just biomass), and fungi (Borowicz 1997, Gehring and Whitham 2002, Bennett et al. 2006). We have previously shown that when ungulate herbivores remove 95% or more of the aboveground biomass of scarlet gilia, the product of lifetime seed production, seed germination, and seedling survival can average as much as 3.0 times that of the uneaten controls, increasing plant fitness (Paige and Whitham 1987, Paige 1992a,b, 1994, 1999, Anderson and Paige 2003). However, the degree of compensation can be affected by environmental conditions such as water limitation (Maschinski and Whitham 1989, Levine and Paige 2004). In particular, Levine and Paige (2004) have shown that drought conditions can adversely impact the compensatory response, through both the direct effects of water limitation on the degree of compensation and the indirect effects of high levels of ungulate herbivory leading to severe undercompensation (Levine and Paige 2004).

In this study, we used a fungicide treatment to significantly reduce fungi and assess their effects on plant fitness following natural patterns of ungulate herbivory. In addition, given that mycorrhizal fungi in particular can improve water relations we were also interested in the interactive role among annual variation in precipitation, fungi, ungulate herbivory and plant compensation. Specifically we addressed the following questions: 1) What is the effect of the fungal community on the compensatory response of scarlet gilia, following ungulate herbivory? Do they facilitate, inhibit or have no effect on compensation; i.e., are they mutualistic, parasitic

or of no consequence? and 2) Is the interactions among ungulate herbivory, mycorrhizal fungi and plant compensation altered under varying climatic conditions (i.e., variation in precipitation)?

Methods and Materials

Study System

Scarlet gilia, *Ipomopsis aggregata*, (Polemoniacea) is a monocarpic biennial/perennial herb growing in western montane regions of the United States (USDA Plants Database). Post-germination, a leafy rosette forms, overwinters, and in the following spring, the majority of rosettes bolt a single paniculate-racemose inflorescence that flower from early-mid July through late September (Paige and Whitham 1985). During the period of stem elongation, mule deer and elk browse, on average, 80% of scarlet gilia plants (Paige 1992a). Over the three years of this study, 83-91% of bolting scarlet gilia plants were browsed (Allsup, unpublished data).

Although scarlet gilia are known to interact with a diverse suite of insect herbivores, including lepidopteran fruit predators (*Heliothis phloxiphaga*: Noctuidae and *Olethreutes* sp.: Tortricidae), a specialist seed fly (*Hylemya* sp.:Anthomyiidae), a stem boring moth (*Sparganothis belfrageana*: Noctuidae), an unidentified green aphid and an unidentified dipteran root borer (Juenger and Bergelson 1998, Anderson and Paige 2003), the high elevation populations that we have been studying in Arizona have been relatively depauperate of insect herbivores. Only green aphids and a lepidopteran fruit predator, *Heliothis phloxiphaga*, have been observed, and at exceptionally low levels (<<1% of plants). Even in a year when *Heliothis* was abundant, there was no significant effect on plant fitness (see Anderson and Paige 2003). Thus our studies have focused primarily on the effects of ungulate herbivory on scarlet gilia.

The fungal community in scarlet gilia roots at this site consists of Ascomycetes dark

septate root endophytes (DSE) characterized by dark septate hyphae and sclerotia, arbuscular mycorrhizal fungi (AMF) characterized by arbusucles (finely-branched tree-like structures) and coenocytic hyphae (lacking cross-walls). In addition, roots were colonized by a small group of non-AMF/DSE fungi including, oospores (Allsup and Paige, this study).

Field Site

Our studies of the interactive effects of scarlet gilia, ungulate herbivory and fungi were conducted within White Horse Meadow in the San Francisco Peaks (altitude approximately 2500 meters) in Coconino County, 17 miles northwest of Flagstaff, Arizona, USA. This population is comprised of more than 30,000 flowering individuals of scarlet gilia located in a montane meadow surrounded by ponderosa pine and aspen. Soils in this area are primarily composed of sand and silt with small amounts of clay. Precipitation, including snowfall, averaged 58.2 cm per year over a 50-year period (NOAA 2007). In the three-year span of this project, two of the three years (2007 and 2009) experienced lower than average precipitation during the growth of scarlet gilia (See Appendix). In 2007, the site experienced lower than average precipitation in late July and August during the flowering period of scarlet gilia and in 2009 it received lower than average precipitation in May and June during the bolting stage of scarlet gilia (2007-27.7 cm and 2009-22.6 cm); both years experienced moderate drought conditions and were among the five driest years over a 59 year history (Hereford 2007)). In 2008, the site approached average precipitation (53.3 cm). All precipitation data were collected from NOAA's (National Oceanic and Atmospheric Administration) National Weather Service station at Pulliam Airport (2129 meters) in Flagstaff, Arizona.

Experimental Design

To assess the effects of belowground fungal associations on the compensatory capability

of scarlet gilia, a factorial experiment was conducted in three years (2007-2009) following herbivory in late May. In 2008 and 2009, a water addition was incorporated. Depending on year, 68-160 bolting plants were randomly selected within a 100 X 50 m grid covering approximately two-thirds of the entire population. The grid was divided into 10 transects spaced 10 m apart and 50 m long. Within each column 6-16 plants (depending on year) spaced approximately 3 m apart treatments were randomly selected; half were naturally browsed and half were unbrowsed. Previous studies (Paige and Whitham 1987, Paige 1992a, 1994, 1999) have failed to show significant differences in fitness between naturally browsed and experimentally clipped plants (whether chosen from the pool of uneaten individuals before or following natural herbivory). On average, 80% (range 64-91%) of all scarlet gilia plants undergo browsing by mule deer and elk in a given year (see below). These results argue that plant selectivity by herbivores had no effect on scarlet gilias' ability to compensate, or alternatively, that herbivores were not selective (Anderson and Paige 2003). Unbrowsed individuals were harder to find and in a few cases, we extended past the grid to achieve the appropriate number of replicates. Plants were then assigned to one of four treatments: (i) unbrowsed controls, (ii) naturally browsed controls, (iii) unbrowsed fungicide-treated plants and (iv) naturally browsed fungicide-treated plants. Overall, sample sizes per treatment ranged from 46-62 (average 57/treatment) in 2007, 20-26 (average 23/treatment) in 2008 to 11-16 (average 14/treatment) in 2009. The smaller sample sizes/treatment in 2008 and 2009 were due to the addition of a watering treatment (see below).

Fungicide treatments were administered to the base of each plant throughout the growing season every two weeks for a total of five treatments. Each plant in fungicide treatments received consisted of 0.5g of the fungicide captan in 280 mL of water. Captan (cis-N-trichloromethyl thio-4-cyclohexane-1, 2-di- carboximide) acts to halt cellular respiration of

fungal organisms and reduce fungal colonization in plant roots (Kough et al. 1987). Each plant in non-fungicidal controls received an equivalent amount of water (280 mL/treatment X 5 treatments = 1400 mL) to mimic the liquid that treatment plants were given.

Following the moderate drought conditions observed in 2007, a water treatment was added to half (160) of all herbivory and fungicide combinations in both 2008 and 2009, given that the compensatory response is dependent upon water availability (Levine and Paige 2004). To assess interactive effects of fungi and water on compensation by scarlet gilia, a total of 1.0 L of water was applied to each plant over a ten-week time span to mimic a 7.5 cm (1.0 L) increase in overall precipitation during the flowering period, totaling 60.8 cm of precipitation in 2008 (10.5 cm during the two and a half month flowering period) and 30.1 cm in 2009 (9.9 cm during the two and a half month flowering period).

Fungicide Efficacy

To test whether fungicide had a non-target impact that could alter soil nutrients, two non-mycorrhizal plant species, Hoary Stock (*Matthiola incana*; Brassicaceae) and Dianthus (*Dianthus chinensis*; Caryophyllaceae), were grown in field soils within site, at the time of the study. Twenty plants of each species were treated with and without a fungicide (representative of the main experiment, see above). In general, plant species in the Brassicaceae and Caryophyllaceae family are considered to be non-host plants for AMF. However, there have been a few contradictory reports of AMF colonization in these two species (DeMars 1995, Gaui & Adholeya 2005). Nonetheless, no fungal colonization was observed in any plant roots of these two species at the end of the study when plants were collected. Biomass of plants with and without a fungicide treatment was compared at the end of the flowering season.

Soil from fungicide-treated and untreated plants was also compared to assess whether

there were differences in soil nutrients. Soils were collected from plants roots following the fifth and final fungicide treatment. Ten randomly selected samples from each treatment were tested for pH, organic matter, available P and K, and N. All chemical analyses were conducted at the A & L Great Lakes Laboratory in Fort Wayne, Indiana.

Plant and Fungal Collections

In September of each year, whole plants from our factorial experiment were collected following senescence. Fine roots were washed free of soil, weighed and transported on ice for storage in a 15˚C cold room. The shoots were dried and assessed for above- and below-ground biomass, and number of fruits. Seed weight was estimated from a randomly drawn subset of ten seeds for each of ten plants collected from each treatment.

Roots were collected to detect presence of belowground fungal structures through microscopic examination of cleared and stained roots. Fungal colonization of roots was assessed on 0.15 grams of fibrous roots from a subset of 10 roots per treatment. Roots were removed and placed in cassettes for clearing using hot 10% potassium hydroxide (Gardner 1975). Roots were then acidified for a stain uptake in a 1% HCl acid solution and stained with a hot 0.05% Direct Blue solution (INVAM 2010). Root colonization was assessed using the "gridline intersection" method at 100 intersections for three separate categories 1) overall AMF colonization (hyphae, arbuscules, vesicles, internal spores), 2) DSE hyphae and sclerotia, and 3) a bin of other fungi under a compound microscope (Giovannetti and Mosse 1980, McGonigle et al. 1990, Brundett et al. 1996) at 400X magnification.

Data Analysis

To assess whether fungicide applications to scarlet gilia had non-target impacts on soil nutrients (N, P, K, OM, and pH), a MANOVA (multivariate analysis of variance) was used to

determine the difference between plants in the fungicide treatment and those in the non-fungicide treatment. In addition, plant biomass of the two non-mycorrhizal species with and without fungicide treatments was compared with an ANOVA (one-way analysis of variance) to evaluate the non-target effects (nutrient availability) of the fungicide treatment.

Root fungal colonization was measured in each year, using a fungicide treatment as an independent variable. Separate ANOVAs were run evaluating the impact of AMF, DSE, and other root fungal colonization, individually, for each year.

We analyzed the compensatory responses of scarlet gilia to the interactive effects of a fungicide treatment, herbivory, and water in ANCOVAs for fruit number, seed mass, aboveground biomass, and belowground biomass over the three years of the study; 2007 (no water treatment), 2008 and 2009 (including a water treatment). In assessing treatment effects, stem diameter was included as a covariate to control for initial size differences of plants. Stem diameter was measured immediately after browsing and matched for size with unbrowsed individuals (we also know that stem scars do not change through time, thus, stem diameter does not respond to treatment; Paige 1994). We employed the MASS function in R for count data. A negative binomial distribution with a logarithmic link, typical of count date, was used to statistically test the total number of fruits per plant (Bolker et al. 2008). All analyses were conducted in R (R Development Core Team 2010.)

Results

Fungicide Efficacy

There was no significant difference in N, P, K, pH, or organic matter content in soils of scarlet gilia plants with or without a fungicide treatment (MANOVA, $p_{fung}=0.86$). The absence of fungicide-induced changes in soil nutrients was further substantiated by the observation that no

significant difference in biomass was detected between non-mycorrhizal plants given a fungicide treatment versus those that were not treated (ANOVA, p_{stock}=0.96, $p_{dianthus}$= 0.69).

Scarlet gilia plants that were administered a fungicide showed a reduction in AMF structures in all three years for browsed and unbrowsed plants, although DSE only showed a trend in reduction 2009 (2007 p_{amf}=<0.001 , p_{dse}=<0.001 , p_{other}= 0.02; 2008 p_{amf}=<0.001 , p_{dse}=0.014, p_{other}=<0.001; 2009 p_{amf}=<0.001 , p_{dse}= 0.116, p_{other}=0.005). Overall, AMF were reduced by 51 $\pm$ 9.73%, DSE were reduced by 69 $\pm$ 47%, and all other fungi were reduced by 75 $\pm$ 35% over the three years of this study (Figure 2.1).

Main Effects of Herbivory, Fungicide and Water on Plant Fitness

Herbivory increased fruit number in all three years (p_{2007}<0.001, p_{2008}=0.008, p_{2009}<0.001, Table 2.1). An, and a significant increase in aboveground and belowground biomass in two of three years (2007 and 2008) for browsed individuals (p_{2007}<0.01, =0.03, p_{2008}<0.01, <0.01, respectively, Table 2.2). Overall, seed mass was significantly greater for browsed plants than unbrowsed plants in 2007 (p=0.04, Table 2.2).

The main effect of fungicide lead to significant overall increases in fruit production in 2007 and 2009 (p_{2007}=0.005, p_{2009}<0.001, Table 2.2). Fungicide treatments also lead to a significant increase in aboveground biomass and seed mass in 2007 (p=0.04, 0.03, respectively, Table 2.2).

Water (assessed only in 2008 and 2009) increased fruit production in both years (p_{2008}=0.02, p_{2009}=0.034, Table 2.2) and a significantly increase aboveground biomass in 2008 (p<0.01, Table 2.2). In addition, water significant decreased in belowground biomass in 2008 (p<0.01, Table 2.2).

Interactive effects of Ungulate Herbivory and Fungi on Compensation

In 2007 and 2009 (under drought conditions), there was a significant interaction between herbivory and fungicide treatments for fruit number and aboveground biomass (Figures 2.2 and 2.4, Tables 2.1 and 2.2). Without a fungicide treatment, plants equally compensated, in terms of the numbers of fruit produced and aboveground biomass (i.e., no significant difference between browsed and unbrowsed plants; ANCOVA, $p_{2007} = 0.78, 0.27$, $p_{2009} = 0.45, 0.68$, respectively) (Figures 2.2 and 2.4). However, with a fungicide treatment, plants overcompensated; browsed individuals produced significantly greater numbers of fruit and greater aboveground biomass compared to unbrowsed individuals (ANCOVA, $p_{2007} = 0.01, 0.04$, $p_{2009} = 0.01, 0.01$, respectively) (Figures 2.2 and 2.4). In contrast, in 2008 (under normal levels of precipitation), there was no significant interaction between herbivory and fungicide on fruit production or aboveground biomass (Figures 2.2 and 2.4, Tables 2.1 and 2.2). Without a fungicide treatment, plants overcompensated in response to herbivory in terms of fruit production and aboveground biomass (ANCOVA, $p_{2008} = 0.012, <0.01$). When fungicide was applied, there was a non-significant trend toward overcompensation (i.e., a trend toward higher fruit production in browsed than unbrowsed fungicide-treated plants; ANCOVA, $p_{2008} = 0.24$). It is important to note that both browsed and unbrowsed plants significantly increased fruit production in 2008 following a fungicide treatment when compared to their natural controls (see Figure 2.2). Aboveground biomass significantly increased for unbrowsed fungicide-treated plants ($p<0.02$) and a non-significant decrease for browsed fungicide treated plants compared to their natural controls ($p=0.24$, Figure 2.4).

Seed mass significantly increased for fungicide-treated browsed plants in 2007 (in comparison to browsed plants without a fungicide treatment; Figure 2.5, ANCOVA, $P=0.05$).

There was a non-significant trend toward higher seed mass for browsed plants in 2009 following a fungicide treatment (Figure 2.5; ANCOVA, P_{2009}=0.23) and a significant increase in seed mass for unbrowsed plants following a fungicide treatment (Figure 2.5; ANCOVA, P_{2009}<0.02). No significant seed mass differences were found in 2008 for browsed or unbrowsed plants following a fungicide treatment (Figure 2.5, Table 2.2, ANCOVA, p>0.05). Belowground biomass was significantly greater in browsed control plants in two (2007 and 2008) of three years (comparing browsed and unbrowsed plants without a fungicide treatment) (Figure 2.6). A fungicide treatment significantly increased belowground biomass for unbrowsed plants in 2007 (p=0.05) and 2008 (p=0.05) (Figure 2.6).

Interactive Effects of Ungulate Herbivory and Water on Compensation

No significant interactions between herbivory and water were found in either 2008 or 2009 for any of the traits measured (number of fruits, above- or below-ground biomass or seed mass) (Tables 2.1 and 2.2). However, in 2008, a water addition significantly increased fruit production in both unbrowsed and browsed plants (Table 1, Figure 2.3A, ANCOVA, p=0.02). In addition, water significantly increased aboveground biomass for unbrowsed plants (p=0.03), significantly decreased belowground biomass for browsed plants (ANCOVA, p=0.03) and had no significant effect on seed mass (p=0.11**,** Table 2.2).

In 2009, a water addition significantly increased fruit production in unbrowsed plants (Figure 2.3B, ANCOVA, p=0.04) with a non-significant trend toward increased fruit production in browsed individuals (Figure 2.3B, ANCOVA, p=0.22). There were no significant effects on above- or below-ground biomass or seed weight (Table 2.2).

Interactive Effects of Ungulate Herbivory, Fungicide and Water on Compensation

In 2008, watered and fungicide treated unbrowsed plants showed a non-significant trend toward increased fruit production over unbrowsed plants with just a fungicide treatment (ANCOVA P= 0.31). Browsed plants given a water and fungicide treatment significantly increased fruit production over browsed plants with just a fungicide treatment (ANCOVA P= 0.05). Both unbrowsed and browsed, water and fungicide treated plants, significantly increased fruit production over unbrowsed and browsed plants with only a water treatment (Figure 2.3A; ANCOVA p=0.04, p=0.05, respectively) and unbrowsed and browsed plants without water or fungicide treatments (Figure 2.3A; ANCOVA, p<0.01, p=0.04, respectively). There was also a significant two-way interaction between water and fungicide and a significant three-way interaction for herbivory, fungicide and water on aboveground biomass (p=0.03 for both two-way and three-way interactions, Tables 2.1 and 2.2). Fungicide generally led to significantly higher aboveground biomass than water additions and the interaction between herbivory, water and fungicide with browsing and fungicide treatments lead to significantly greater effects on aboveground biomass than water treatments.

In 2009, water and fungicide treated unbrowsed plants showed a non-significant trend toward increased fruit production over unbrowsed plants with just a fungicide treatment (Figure 2.3B, ANCOVA, p=0.36). When browsed plants were given both a fungicide and a water treatment, fruit production significantly increased over those with just a fungicide treatment alone (Figure 2.3B, ANCOVA, p=0.03). Water and fungicide treated browsed plants significantly increased fruit production over browsed plants with only a water treatment (Figure 2.3B, ANCOVA, p=0.03). No significant differences were found on fruit production for water and fungicide treatment on unbrowsed plants when compared to water only treated unbrowsed

plants (Figure 2.3B, ANCOVA, $p=0.68$). Both unbrowsed and browsed water and fungicide treated plants produced significantly more fruit than untreated controls (Figure 2.3B; ANCOVA, $p<0.01$, $p<0.01$, respectively).

Discussion

In this study we were interested in how fungal associations affected the compensatory response of scarlet gilia, *Ipomopsis aggregata*, following ungulate herbivory. We hypothesized that fungal associations would facilitate the compensatory response most commonly observed in this Arizona population of scarlet gilia; perhaps mutualistic associations with fungi, such as arbuscular mycorrhizal fungi (AMF), would explain the phenomenon of overcompensation altogether. However, fungal removal experiments indicate that fungi are not mutualistic but parasitic, particularly under the combined effects of herbivory and drought. Fungicide-treated browsed plants produced 1.2 – 1.9 times as many fruit as browsed plants without a fungicide treatment over the three years; 1.7-1.9 times as many fruit under drought conditions. However, fungicide-treated unbrowsed plants showed no significant increase in fruit production in either 2007 or 2009, but significantly increased fruit production in 2008 over unbrowsed plants without a fungicide treatment. Specifically, drought conditions appear to limit the capacity of unbrowsed plants to respond to the fungicide treatment. This is substantiated by the fact that when unbrowsed plants received both fungicide and water treatments in a drought year (2009), there was a significant increase in fruit production over unbrowsed control plants. In contrast, browsed plants increased fruit production whether they experienced drought conditions or not following a fungicide treatment. In addition, the interactive effects of herbivory, fungicide treatment and water additions resulted in even greater compensatory capabilities in terms of fruit

production, whether under drought conditions or environmentally favorable conditions (normal levels of precipitation).

These results lead one to ask why there are differences between browsed and unbrowsed plants in their fitness responses following a fungicide treatment. Recent studies on *Arabidopsis thaliana* in our lab have shown that differences in the degree of endopolyploidy (i.e., plasticity in cellular ploidy through endoreduplication) following the removal of apical dominance, leads to enhanced reproductive success, i.e., overcompensation (Scholes and Paige 2011, Scholes et al. 2013, Scholes and Paige 2014). *Ipomopsis aggregata* also plastically endoreduplicates following the removal of apical dominance (Paige, unpublished data) Thus we suspect that it responds similarly to *Arabidopsis* from a molecular genetic perspective. Increasing chromosome number, and thus gene copy number, may provide a means of increasing gene expression, likely by the up-regulation of selected genes or gene families. We have recently shown that Glucose-6-Phosphate Dehydrogenase 1, the key regulatory enzyme in the Oxidative Pentose-Phosphate pathway that plays a central role in plant metabolism converting glucose to ribose-5-phosphate, significantly up-regulates following the removal of apical dominance in genotypes that overcompensate (Siddappaji et al. 2013). Furthermore, increasing chromosome number increases the total DNA content and hence cell size leading to extensive cell growth/expansion through endoreduplication. Hence greater demand by plants for nutrients and water following herbivory and greater nutrient and water transport as a consequence of endoreduplication, likely explain the differential responses of browsed and unbrowsed plants to the removal/reduction of fungi. In addition, root biomass also generally increased following browsing, initially acting as a sink for carbon from the newly regenerating tissues. The increase in root biomass and spread in both the tap and fibrous portions of the root system may further facilitate the accumulation and transport

of nutrients and water contributing to enhanced aboveground biomass and fruit production. On average, seed mass increased for both browsed and unbrowsed plants following a fungicide treatment in the two drought years. We hypothesize that there is a tradeoff between producing few large seeds per fruit versus producing many small seeds, with larger seeds having a survival advantage under more severe drought conditions due to greater oil and water stores. Unfortunately, seed number per fruit was not assessed in this study to test this idea.

Our results are counter to the "modification of tolerance hypothesis" proposed by Bennett et al. 2006 that plants associating with mycorrhizal fungi will have higher tolerance to herbivory. It is likely that AMF and DSE's are competing with plants for nutrients, carbon and water following herbivory (Johnson, Graham and Smith 2004, Johnson et al. 1997, Jones and Smith 2004, Hoeksema et al. 2010), driven in part by water limitation. Plants only equally compensated under the drought conditions of 2007 and 2009, but overcompensated following a fungicide treatment, reducing their fungal competitors. In a year of normal precipitation plants naturally overcompensated and significantly increased fruit production following a fungicide treatment in both browsed and unbrowsed plants with a non-significant trend toward overcompensation. Despite the low levels of non-AMF/DSE colonization (stain may vary in its ability to detect all pathogenic fungi inhabiting plant roots), root-infecting pathogenic fungi (oospores) may impact overall plant growth. However, a comparison of plant fitness following herbivory with and without pathogenic fungi showed no effect of these fungi on fitness in any of the three years of study (p_{2007}=0.65, p_{2008}=0.39, p_{2009}=0.08). Variation in the level of colonization could also play a role in constraining the compensatory response following herbivory. For example, Garrido et al. 2010, showed a negative correlation between plant tolerance to defoliation in *Datura stramonium* and AMF colonization levels.

Soil moisture and herbivory may also interact to influence AMF species composition (Murray et al. 2010). For example, fungal species that colonize roots following browsing may differ from those that colonize plants that are not browsed. Individual species of AMF are known to differ in their effects on plant growth, ranging from mutualistic to antagonistic (Johnson 1997, Klironomos 2003, Bennett et al. 2006). Thus, the outcome of any interaction may be contingent on the particular fungal species that colonize roots following browsing (Bennett and Bever 2007). Additional studies will be necessary to address how different AMF species differentially affect the compensatory response.

Overall, our results indicate that the fungal community strongly influences the compensatory response of scarlet gilia and the magnitude of the response is dependent upon environmental conditions (in this case water). Experimental water additions alone did not affect the compensatory response, although water additions did increase plant fitness, generally. The reduction in fungi, however, did alter the compensatory response particularly under water-limited conditions, increasing compensation from equal to over compensation. In a year of normal precipitation, a fungicide treatment reduced the compensatory response from overcompensation to a marginally significant trend toward overcompensation, by simultaneously increasing the reproductive success of both browsed and unbrowsed plants. We also show experimentally that the interactive effects of water and fungicide maximize fruit production following herbivory. Thus, fungi are clearly parasitic on scarlet gilia, particularly following ungulate herbivory.

This study represents one of few to consider the interactive effects ungulate herbivory and fungal associations on plant fitness and represents the only study addressing the phenomenon of overcompensation. It also represents one of few studies focusing on plant reproduction as

opposed to biomass alone. Aboveground biomass and fruit production were at best weakly correlated (R^2=0.29, p=0.001), thus biomass may not give a clear picture of changes that are ultimately of evolutionary importance.

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Tables and Figures

Table 2.1. Effects of herbivory, fungicide and water treatments (in 2008 and 2009) on fruit production in *I. aggregata*. Data were analyzed using a negative binomial distribution with a logarithmic link. Stem diameter is used as the covariate to control for size.

		2007		2008		2009	
	df	Z	P	z	P	*z*	P
Herbivory	1	-3.55	**<0.001**	-2.66	**<0.01**	-5.03	**<0.001**
Fungicide	1	-2.78	**<0.01**	-1.66	0.25	-3.72	**<0.001**
Stem	1	4.75	**<0.001**	5.01	**<0.001**	5.43	**<0.001**
Water				2.3	**0.02**	0.96	**0.03**
H X F	1	1.96	**0.05**	-0.61	0.55	2.2	**0.03**
H X W				-0.32	0.75	0.86	0.39
F X W				-0.99	0.32	-0.06	0.95
H X F X W				0.37	0.71	0.5	0.98

Notes: z-values (z) with significance levels (P) and degrees of freedom (df) are given.

Table 2.2. Effects of herbivory, fungicide and water treatments (2008 and 2009) on attributes of plant fitness (seed mass (mg), aboveground biomass (g), and belowground biomass (g)) in *I. aggregata*. Data were analyzed with an ANCOVA. Stem diameter was used as the covariate to control for size.

	2007			2008			2009		
Above biomass	1. df	F	P	df	F	P	df	F	P
Herbivory	1	17.06	**<0.01**	1	12.83	**<0.01**	1	0.84	0.36
Fungicide	1	4.47	**0.04**	1	3.39	0.07	1	0.03	0.86
Stem	1	49.54	**<0.01**	1	22.88	0.82	1	50.01	**<0.01**
Water				1	0.05	**<0.01**	1	2.08	0.15
H X F	1	4.6	**0.03**	1	0.07	0.79	1	4.66	**0.03**
H X W				1	1.33	0.25	1	2.68	0.1
F X W				1	4.6	**0.03**	1	1.06	0.31
H X F X W				1	4.79	**0.03**	1	2.62	0.11
Error df	227			184			101		
Belowground biomass	Df	F	P	df	F	P	*df*	F	P
Herbivory	1	4.66	**0.03**	1	7.57	**<0.01**	1	0.12	**0.73**
Fungicide	1	1.8	0.18	1	2.6	0.11	1	0.22	0.64
Stem	1	59.77	**<0.01**	1	5.26	**0.02**	1	23.43	**<0.01**
Water				1	27.54	**<0.01**	1	<0.01	0.98
H X F	1	2.91	0.09	1	0.13	0.72	1	0.54	0.54
H X W				1	2.87	0.09	1	0.07	0.07
F X W				1	3.35	0.07	1	0.91	0.91
H X F X W				1	0.73	0.40	1	0.27	0.27
Error df				184			101		
Seed mass	Df	F	P	df	F	P	*df*	F	P
Herbivory	1	4.38	**0.04**	1	0.02	0.90	1	0.55	0.46
Fungicide	1	4.6	**0.03**	1	0.02	0.88	1	2.62	0.11
stem	1	0.25	0.62	1	0.04	0.84	1	0.03	0.86
Water				1	9.37	0.11	1	2.1	0.15
H X F	1	1.07	0.3	1	3.05	0.08	1	0.1	0.75
H X W				1	0.6	0.44	1	0.19	0.66
F X W				1	0.07	0.79	1	3.9	**0.05**
H X F X W				1	0.3	0.58	1	1.36	0.25
Error df	121			184			101		

Notes: F-values (F) with significance levels (P) and degrees of freedom (df) are given.

Figure 2.1. Measurements of overall fungal colonization +/- S.E. in *Ipomopsis aggregata* roots with and without ungulate browsing and fungicide treatments in each year (2007, 2008, and 2009) of the study.

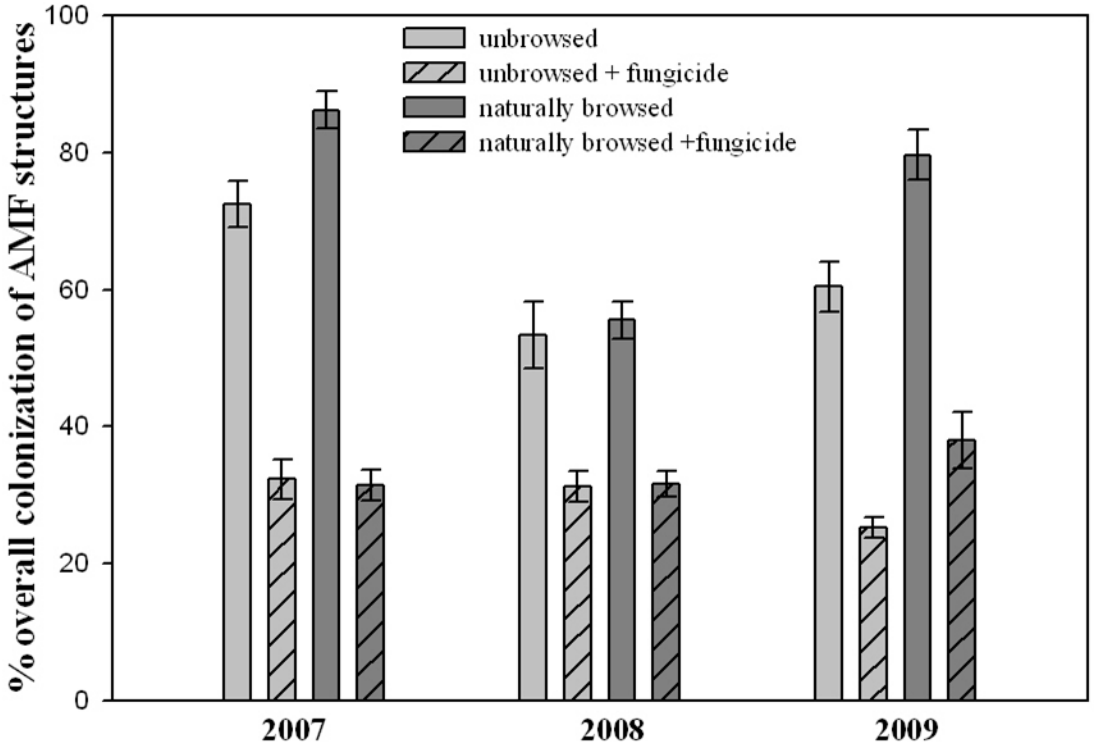


Figure 2.2. Average fruit number +/- S.E. of *Ipomopsis aggregata* plants with and without ungulate browsing and fungicide treatments in each year (2007, 2008, and 2009) of the study.

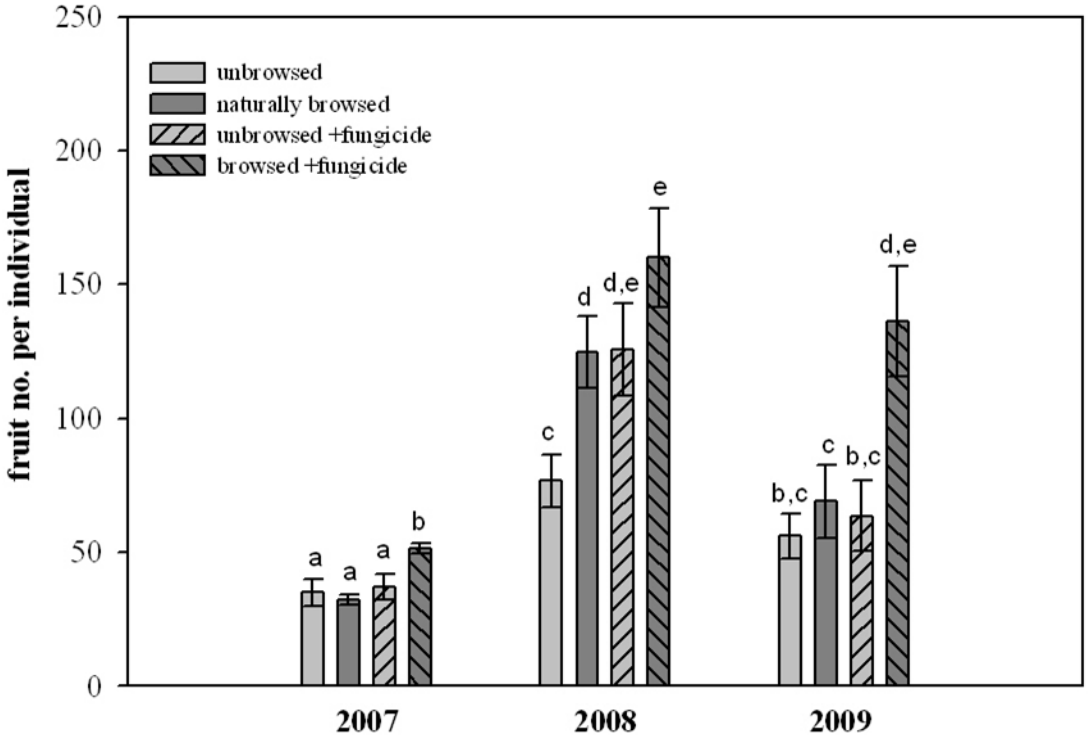


Figure 2.3. Average fruit number +/- S.E. of *Ipomopsis aggregata* plants with and without ungulate browsing, fungicide and water treatments in 2008 (A) and 2009 (B).

A.

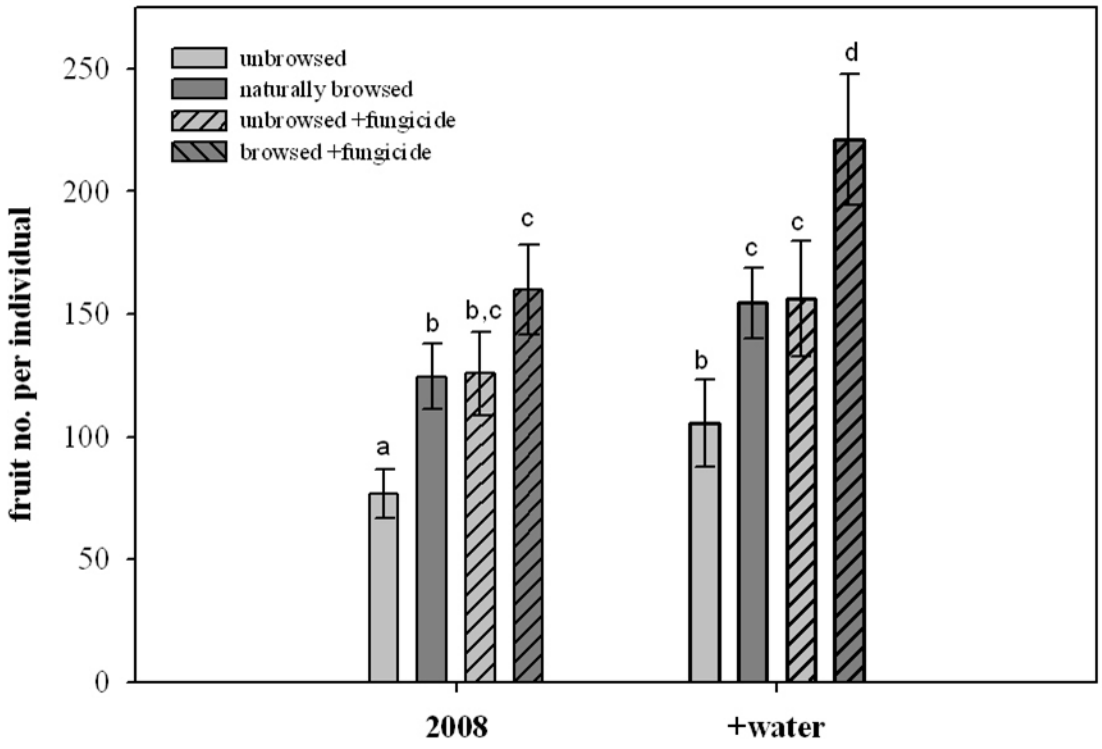


Figure 2.3 (cont.)

B.

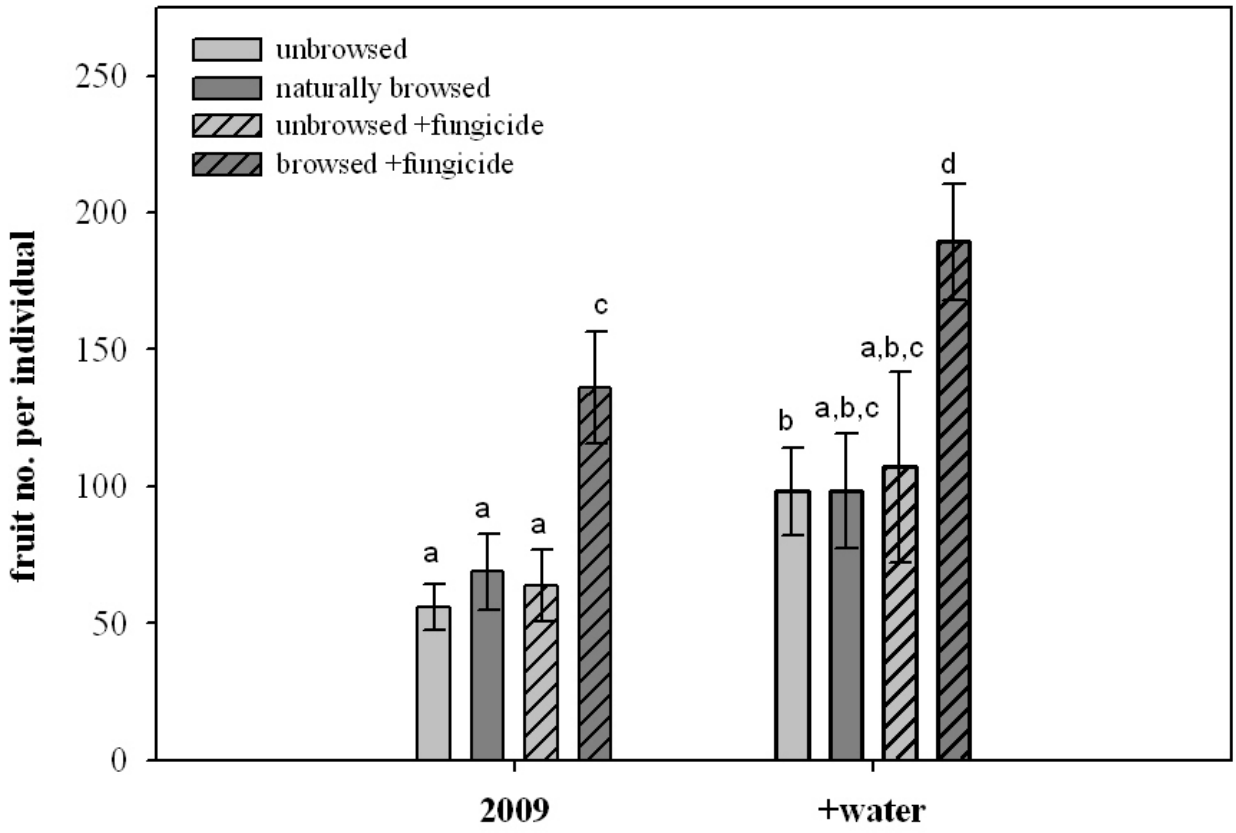


Figure 2.4. Measurements of *Ipomopsis aggregata* aboveground biomass +/- S.E. with and without browsing and fungicide treatments for 2007, 2008 and 2009.

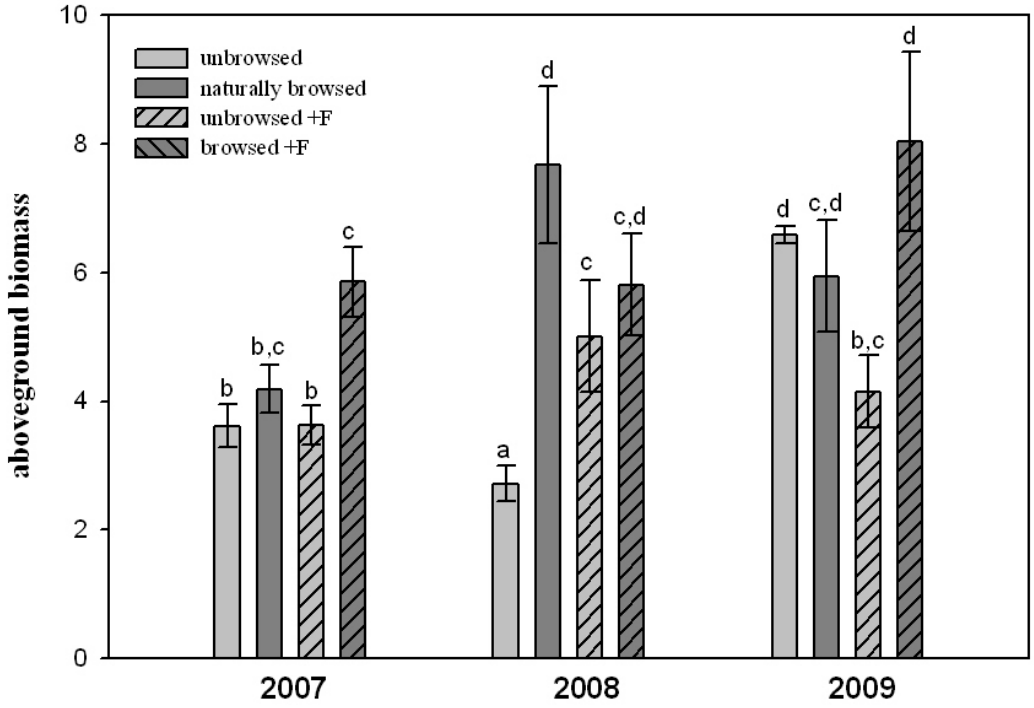


Figure 2.5. Measurements of *Ipomopsis aggregata* seed mass +/- S.E. with and without browsing and fungicide treatments for 2007, 2008 and 2009.

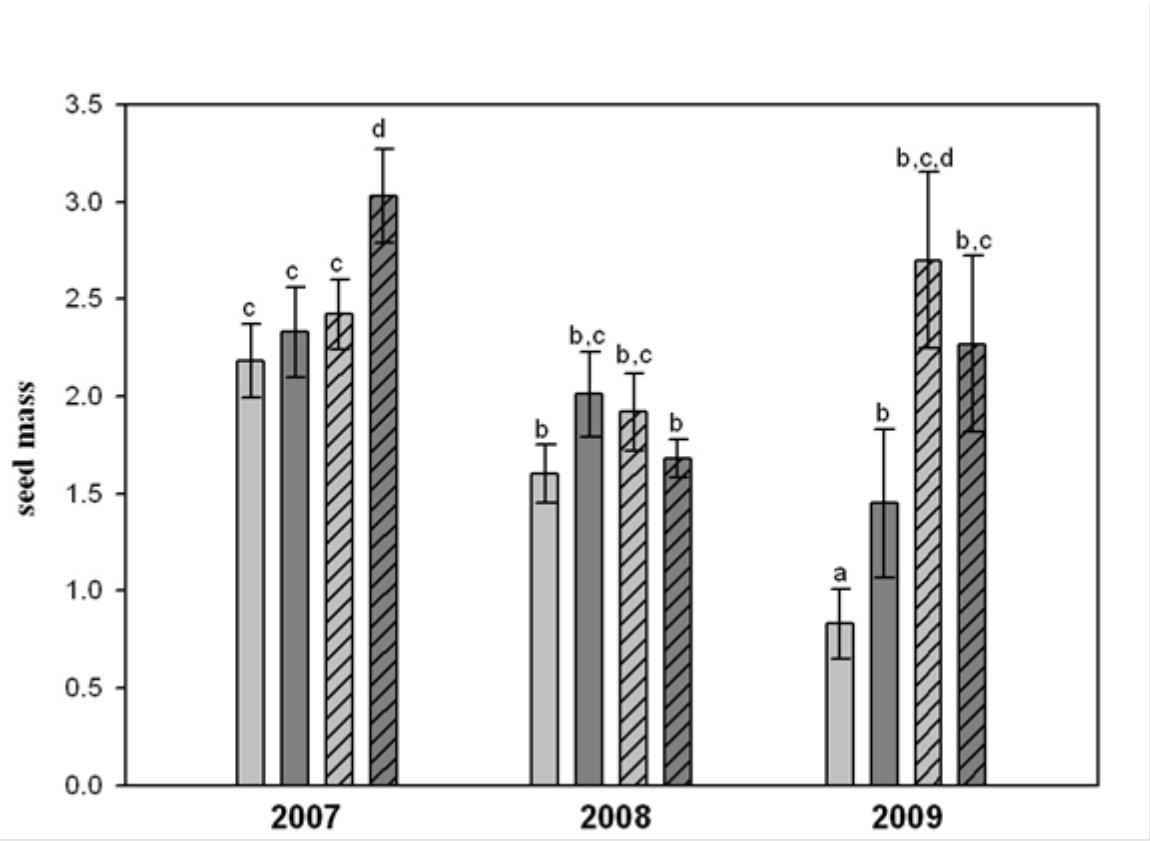
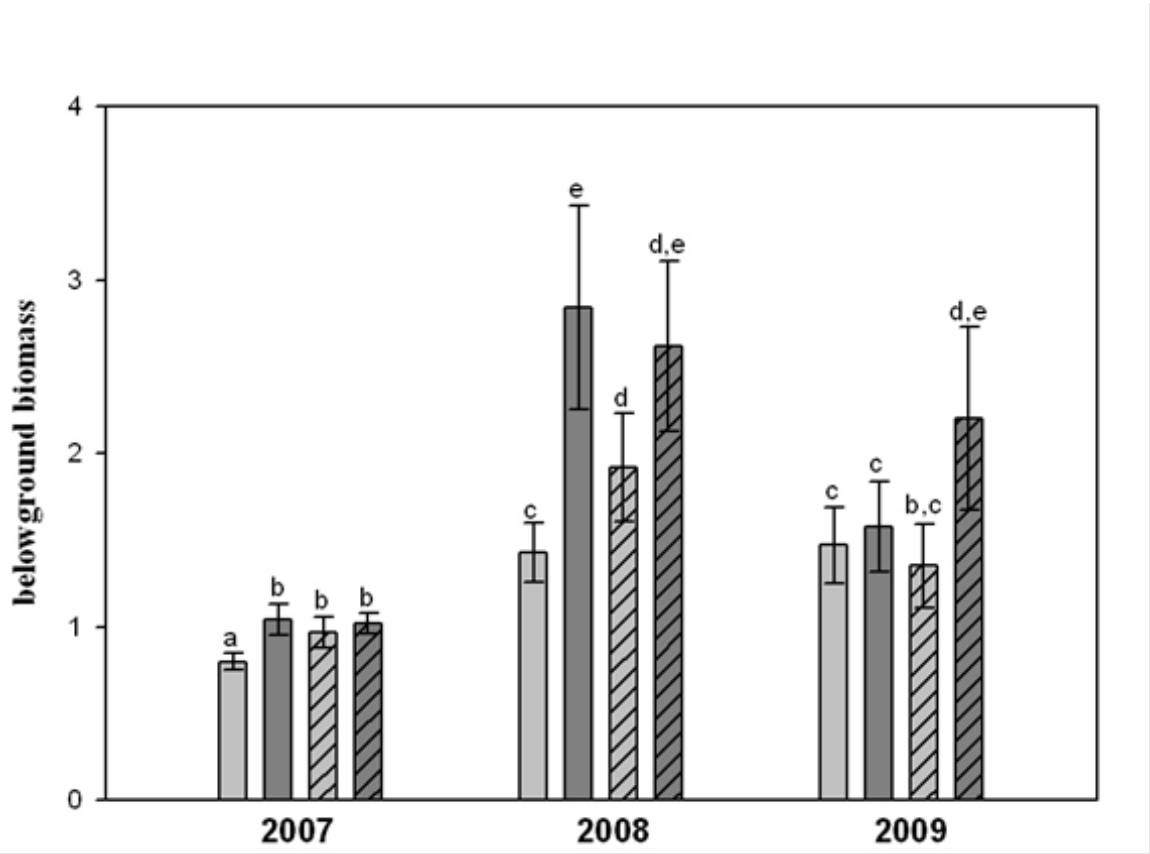


Figure 2.6. Measurements of *Ipomopsis aggregata* belowground biomass +/- S.E. with and without browsing and fungicide treatments for 2007, 2008 and 2009.



CHAPTER 3: THE INTERACTIVE EFFECTS OF HERBIVORY AND WATER ON ARBUSUCLUAR MYCORRHIZAL FUNGAL COLONIZATION, COMMUNITIY COMPOSITIONS AND FITNESS COMPENSATION

Abstract

Herbivory and water availability have been well established to be determinants of plant growth in montane meadows. However, less documented is the impact of these factors on the composition of soil fungi and how they potentially feedback on plant regrowth and fitness compensation. It has been previously shown that the soil fungal community negatively affects the compensatory response of *I. aggregata*, particularly under drought conditions. Here, the impacts of water availability and herbivory on fungi in plant roots and rhizosphere soils were assessed using a multifactorial field experiment. Following plant harvest, intraradical colonization and fungal community differences were assessed in plant roots and rhizosphere soils.

Results show that both water availability and herbivory had significant effects on levels of fungal colonization and arbuscular mycorrhizal fungal (AMF) species distribution patterns. Specifically, drought conditions enhanced overall levels of fungal colonization of both AMF and dark septate endophytes (DSE). In addition, ungulate herbivory, lead to higher colonization of AMF hyphae and arbuscules under drought conditions. It is likely that under drought conditions, fungi, AMF colonized browsed plants at higher levels due to the root becoming a sink for carbon, generated by the newly juvenalized regrowth tissue following the release of apical dominance. Ungulate herbivory also enhanced, or tended to enhance, the richness and diversity of the AMF community in roots and soils. An increase in species following browsing, particularly *Scuttellospora* sp. may indicate that specific fungal species are responsible for the parasitic response of the soil fungal community on the compensatory response of *I. aggregata.* These

results suggest that soil fungal community loads and fungal species dictate the magnitude of fitness compensation following ungulate herbivory.

Introduction

Non-pathogenic fungal communities colonize a majority of terrestrial plant roots. In general, arbuscular mycorrhizal fungi (AMF) form symbiotic relationships with their host plants, in which the fungi trade nutrients and water through an extended hyphal network for photosynthetic products. Plant-AMF relationships are important in all aspects of plant ecology, from individual plant performance (van der Heijden and Sanders 2002, Hartnett & Wilson 2002, Facelli et al. 2010, Klironomos et al. 2011) to population and community dynamics (Hartnett and Wilson 2002, Smith et al. 2010) and, ultimately overall ecosystem function (Maherali & Klironomos et al. 2011, Johnson et al. 2012). Up to 80% of all plants growing in native environments can be colonized by several species of AMF. The growth response of the host can vary from positive to negative with different plant-fungal combinations and environmental conditions (Johnson et al. 1997, van der Heijden and Sanders 2002, Klironomos 2003, Smith and Read 2008). Thus the long-held notion that plant-mycorrhizal interactions are solely mutualistic does not hold true (e.g., see Chapter 2).

Another important and less studied group of root fungi are the dark septate endophytes (DSE). Dark septate endophytes are a miscellaneous, understudied, group of Ascomycetes that could form beneficial relationships with the host plant, primarily by protecting plants from soil pathogens (Jumponnen and Trappee 1998). Similar to AMF, DSE can lie along a mutualism-parasitism continuum (Johnson et al.1997, Jumpponen 2001).

Plant-soil fungal interactions are primarily studied from the host plant's perspective; consequently, we know relatively little about how interacting external forces affect AMF/DSE

success/fitness (Smith and Read 2008). Herbivory and water availability, for example, have been shown to alter relationships between mycorrhizal fungi and their hosts because of the nutrient/water requirements for host regrowth and the provisions of C supplied to the fungal species (Gehring and Whitham 2002). In most cases, herbivory has been found to reduce AMF colonization and change community composition (Gehring and Whitham 2002, Murray et. al 2010). Herbivory has also been noted to change fungal structural allocation inside plant roots (presence of hyphae, vesicles, arbuscules, and internal spores), resulting in negative impacts on AMF symbiosis (Wearn and Gange 2007, Murray et al. 2010). For example, a decline in arbuscules (nutrient exchange site between host plant and AMF) may decrease the amount of P given to the plant by the fungi and, in return, decrease the amount of carbon given to the fungi by the plant (Smith and Read 2008). Drought conditions may improve the AMF symbiosis, enabling plants to receive more water due to extramatrical hyphae extension into soils that are not within reach by plant roots (Allen 2007, Augé 2001, Augé 2004).

In northern Arizona, ungulate herbivory on *Ipomopsis aggregata* can lead to increased plant fitness (termed overcompensation) (Paige and Whitham 1987). Overcompensation is primarily observed when water is not a limiting factor (Levine and Paige 2004, Chapter 2). In this system, soil fungal communities had negative effects on compensatory growth, as their removal lead to overall increases in fitness for browsed plants (and unbrowsed plants when water was not a limiting factor; Chapter 2). The combinatorial effects of both herbivory and drought to the plant altered the compensatory outcome that might be dependent on root allocation of AMF hyphae, AMF arbuscules, AMF vesicles and AMF spores, and DSE and/or the AMF taxa involved. A multi-factorial field experiment was performed that addressed the combined impact of herbivory

and water availability on the abundance and community composition of fungi in both plant roots and rhizosphere soils.

Materials and Methods

Study organisms

Scarlet gilia, *Ipomopsis aggregata*, is a biennial, monocarpic herbaceous plant that is abundant in montane meadows of the western United States (USDA plant database). Their first year is spent as a vegetative rosette and in the second year a single paniculate inflorescence bolts in early spring, flowering by mid-late July- early August. During the spring of 2008 and 2009, mule deer (*Odocoileus hemionus*) and elk (*Cervus canadensis*) browsed 73% and 91% of plants, respectively (Allsup, personal observation).

Fungal community in scarlet gilia roots consists of approximately 60-90% colonized by AMF and 1-4% colonized by DSE (Chapter 2). All other fungi represent less than 1% colonization (Chapter 2). Arbuscular mycorrhizal fungi (AMF) characterized by arbuscules (finely-branched tree-like structures) and coenocytic hyphae (lacking cross-walls) and ascomycetes dark septate root endophytes (DSE) characterized by dark septate hyphae and sclerotia. In addition, roots were colonized by a small group of non- AMF/DSE fungi including oospores.

Study Site

Experiments were conducted in Coconino County, 20 miles northwest of Flagstaff, Arizona, USA (altitude approximately 2500 meters). This population averaged 30,000 flowering individuals of scarlet gilia surrounded by ponderosa pine (*Pinus ponderosa*) and aspen (*Populus tremuloides*). In 2008, the site experienced average precipitation levels equaling 53.3 cm. (NOAA 2008). In 2009, the majority of the growing and flowering period experienced moderate drought

conditions with overall precipitation equaling 27.7 cm (-27.6 cm below average precipitation) (NOAA 2009).

Experimental design

To assess the interactive effects of herbivory and water availability on belowground fungal associations, a factorial experiment was conducted in late May of 2008 and 2009. Plants were assigned to one of four treatments: (i) unbrowsed controls, (ii) naturally browsed controls, (iii) unbrowsed water-treated plants and, (iv) naturally browsed water-treated plants. For this experiment, 68 and 160 bolting plants in 2008 and 2009, respectively, were randomly selected within a 200 X 50 m grid covering approximately two-thirds of the entire population. The grid was divided into 20 transects spaced 10 m apart. Within each transect, plants were selected every 3 m. Half were naturally browsed and half were unbrowsed. Unbrowsed individuals were harder to find in a few cases. Thus, we extended past the grid to achieve the appropriate number of replicates. A water treatment was added to half of all plants. To each plant in the water treatment, 1.0 L of water was applied across a ten-week period to mimic a 7.5 cm increase in overall precipitation. The total precipitation for each year and treatment was 53.3 cm in 2008, 60.8 cm following water additions in 2008, 27.7 cm in 2009 and 35.2 cm following water additions in 2009.

In September of each year, whole plants were carefully collected prior to senescence. Aboveground biomass and roots were separated in the field. Aboveground plant material was transferred to paper bags, allowed to dry, and shipped to the University of Illinois for weighing and counting fruit. Entire root balls were, packed on ice, and transported on ice to the Northern Arizona University field station. Rhizosphere soils from the root balls were sieved and roots were

washed free of soils. Both were shipped to Illinois on dry ice. Soils remained in a 15˚C cold room up to 10 days and roots were frozen at -80 ˚C.

Assessment of fungal associations in plant roots

To detect the presence of belowground fungal associations in plant roots ten roots per treatment were cleared of cellular content and stained for examination under a microscope at 1000X magnification (Giovannetti and Mosse 1980, McGonigle et al. 1990, Johnson et al. 1999). Fungal colonization was measured on 0.15 grams fibrous roots. Roots were placed in cassettes for clearing using hot 10% potassium hydroxide (Gardner 1975), acidified for a stain uptake in a 1% HCl solution and stained with a hot 0.05% Direct Blue solution (INVAM 2010). Root colonization was assessed using the "gridline intersection" method for 100 intersections from which percentages of AMF hyphae, AMF arbuscules, AMF vesicles, AMF internal spores, and DSE (s, dark septate endophytes). DSE are less able to uptake chitin-adhering dyes, thus, their abundance is likely an underestimate of their true abundance (Barrow and Aaltonen 2001, Mandyam and Jumpponen 2005).

The structure of the AMF community was determined by 18S rRNA gene sequences. To characterize species composition occupying plant roots, comparison of peak profiles from TRFLP (Terminal Restriction Fragment Length Polymorphism) was used for all herbivory (naturally browsed, unbrowsed), water treatment combinations, and year (2008-2009). We sampled eight plants per treatment per year.

DNA was extracted from plant roots using the OMEGA EZ 96 Plant DNA Spin Kit (Omega Bio-tek, Atlanta, GA, USA) and amplified an ~800 bp fragment of the 18S rRNA gene using a nested PCR protocol (Lee et al. 2008, Lankau and Nodurft 2013). The first reaction was carried out using 1 ul of undiluted DNA, 1.25U Taq polymerase, and 0.6 ug T4 in 1X reaction

buffer, including a concentration of 1.5 mM MgCl, 20 uM of each dNTP, and 0.4 uM of each of the general eukaryote NS1-NS4 primers (White et al. 1990). PCR was performed with the following protocol: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 40°C for 1 min, 72°C for 1 min, and a final extension period of 72°C for 10 min. For the second reaction, 0.5 ul of the NS1-NS4 PCR product was used as atemplate in order to amplify the Glomeromycota specific DNA regions delimited by the AML1-AML2 primers (Lee et al. 2008.), using a touchdown procedure to reduce non-specific amplification. We used 10 uL reactions containing 1.25U Taq polymerase and 7 ug BSA in 1X accompanying buffer with concentrations of 1.5mM MgCl, 20 uM of each dNTP, and 0.4 uM of both AML1-FAM and AML2-NED. The PCR cycling program was as follows: 94°C for 15 min, followed by 35 cycles of 94°C for 30 s, 65°C-58°C (temperature drops of 0.2°C each cycle for 35 cycles) for 40 s, 72°C for 55 s and a final extension at 75°C for 5 min.

In previous studies this protocol has been successful in amplifying AMF DNA with very little non-specific amplification (Lankau and Nodurft 2013). PCR products were digested with MboI (Promega) following the manufacturer's protocol, and a 1:4 dilution of digested product was sized via capillary electrophoresis. T-RFLP peaks were analyzed with Genemarker, and peaks were collapsed into "identity bins" based on previous analysis of cloned AMF 18S DNA from taxa across the Glomermycota phylogeny (see Lankau and Nodurft 2013 for details).

Assessment of fungal spore distribution in rhizosphere soils

Root and soil analyses were employed to obtain the best representation of the entire AMF community present (van der Heijden et al. 2002), given that some AMF identified in roots can be underrepresented in soils and vice versa (Clapp et al. 2003, Dickson 2004). A total of 60 g of soil was used for 10 replicates per treatment combination per year; 30g designated to calculate spore

abundance and 30g for inoculum to create trap cultures (trap culture methodology described below). AMF spores were isolated using the wet sieving and decanting method followed by sucrose centrifugations (Daniel and Skipper 1982, McKenny and Lindsey 1987, Johnson et al. 1999). Following centrifugation, the supernatant was poured through a 25 um and 250 um sieve and rinsed with tap water (Johnson et al.1999). Spores were transferred to a gridded petri dish and viewed underneath a dissecting scope at 1000X magnification and total abundances were determined by total number of spores.

Trap cultures were established with the remaining 30 g of field-isolated AMF spores within 10 days of collecting native soils. Trap cultures were established from indigenous AMF spores to obtain viable non-degraded specimens for easy identification (Johnson 1999, INVAM). Isolated spores and 0.15g of root were chopped into small fragments and mixed with an autoclaved media (1:1 Az site soils and coarse sand). The mixture was transferred to 13.1 cm deep cone-tainer planting tubes and planted with surface sterilized seeds of *Sorghum bicolor* and *Zea mays*. It is important to note that it is probable that there is host specificity of bait plants to AMF species (Klironomos 2004). Each was allowed to germinate and grow for up to three months. Plants were grown in a greenhouse with temperatures ranging from 20-25°C and relative humidity ranging from 65-75%. Plants were watered every two days with tap water. After 3 months, the pot cultures were sampled by taking 30 g of soil, storing them in a 5°C cold room for one week, followed by spore extraction (as noted above) and identification. Spores were grouped according to morphological characteristics (spore size and color, wall structure and hyphal attachment) described in the INVAM database (available online).

Assessment of fungal associations in plant roots

A MANOVA (Multivariate of Analysis of Variance) was used to test whether overall colonization of AMF differed among browsing and water treatments, year, and their interactive effects. Once a significant MANOVA was found, post-hoc ANOVAs were used to test for browsing and water treatment differences in % colonization for AMF hyphae, AMF arbuscules, AMF vesicles, AMF spores, DSE, and a bin on non-AMF/DSE fungi for each year.

AMF richness was determined in roots as the number of T-RFLP bands in each sample. This is a minimum because multiple species can potentially produce identical bands using our protocols (Lankau and Nodurft 2013). The Shannon-Weiner diversity of each sample was calculated using the fluorescence intensity of each TRF as a measure of abundance and using the vegan package in R (Oksanen 2005). All analyses using fluorescence intensity as a measure of abundance are potentially biased by differential PCR amplification of taxa and so these analyses must be treated with caution.

A generalized linear model with a Poisson error distribution was used to test differences in AMF richness among browsing and water treatments and their interactions, separately for 2008 and 2009. Significance of model terms was determined using Likelihood Ratio Tests of nested models (with and without the term in question). The estimated dispersion parameter for the Poisson regressions was substantially less than one (~0.35), suggesting that the data may not be well described by a Poisson distribution. Therefore, results are conservative as the model assumes a greater variance than data show. An ordinary least squares regression was used to compare Shannon-Weiner diversity among treatments. In both analyses, the total fluorescence (sum of fluorescence intensities of all detected peaks in a sample) was included as a covariate to

account for differences in PCR efficiency, which may act similar to in sampling intensity (i.e. one expects to detect more species with more individuals sampled).

Assessment of fungal spore distribution in rhizosphere soils

A GLM with a Negative Binomial Distribution was used to determine the impacts of browsing, water treatments, and their interaction on rhizosphere spore counts from field soils in each year. The MASS function in R was utilized for count data. Planned contrasts were performed using post-hoc analysis of variance (ANOVA)s to examine impacts of treatments and year.

For trap-cultured spores a GLM with a Negative Binomial distribution was used to test for differences in AMF richness differed among browsing treatment, water treatment, and their interaction in each year. And, an ANOVA was used for differences among a browsing treatment, water treatment, and their interactions in each year. We compared compositions using relative abundance of each species. ANOVAs were employed to determine the significance of browsing , water treatments, and their interaction on each of the five identified species (*Glomus intraradices, Paraglomus occulutum, Glomus sp., Scutellospora calospora, Scuttellospora pellucida*) in each year.

Results

AMF colonization and composition in roots

Results showed significant effects of herbivory, water additions, and their interaction, and on root colonization by AMF (MANOVA, $p<0.001$, Table 3.1). Notably, root samples from 2009 had significantly higher percentages of AMF hyphae, arbuscules, vesicles and internal spores than 2008 (except for the watering treatments comparing vesicles which showed no significant differences between 2008 and 2009) (Table 3.1, Figure 3.1A). In addition, to generally lower

AMF colonization levels in 2008, herbivory alone resulted in a significant decrease in hyphal colonization but no significant effects on arbuscules, vesicles or internal spores (Table 3.1, Figure 3.1A). In contrast, herbivory led to an increase in arbuscules and hyphae and a reduction in the presence of vesicles in 2009 (Table 3.1, Figure 3.1A).

Water additions significantly increased the percentage of arbuscules and internal spores for both unbrowsed and browsed plants in 2008 (Table 3.1, Figure 3.1A), with a significant interaction between herbivory and water for internal spores. In 2009, water additions significantly increased the percentage of arbuscules for both unbrowsed and browsed plants and hyphae for unbrowsed plants (Table 3.1, Figure 3.1A). Water additions also interacted with browsing, by decreasing vesicles and increasing internal spores (Table 3.1, Figure 3.1A).

On average, DSE comprised 1 to 3.4% of total colonization (Table 3.1B, Figure 3.1B). DSE increased colonization rates from .33% in average conditions (2008) to 2.19% in drought conditions (2009) (Table 3.1, Figure 3.1B).

AMF diversity was higher in browsed than unbrowsed plant roots in 2008, with no significant differences in 2009 (P_{2008}=0.02, P_{2009}=0.74, Figure 3.2). Water additions had no significant effect on diversity in either year (P_{2008}=0.3, P_{2009}=0.17, Figure 3.2). AMF richness, showed similar qualitative trends, but effects were only marginally significant (P_{2008}=0.12, P_{2009}=0.72, Figure 3.2). The browsing X water interaction was not significant for either diversity or richness in either year.

AMF spore abundance and community composition in soil

Spore abundance was 1.6-fold greater in drought conditions of 2009 compared to 2008, a year of normal precipitation (Figure 3.3). Browsing significantly increased AMF spore abundance in both years by approximately 2-fold (Table 3.3, Figure 3.3). Under drought conditions in 2009,

water increased spore number by approximately 1.2-1.5 fold, but there was no significant impact of water on spore number in 2008 (Table 3.3, Figure 3.3). There was a marginally significant interactive effect between herbivory and water under the drought conditions of 2009 (P=0.08), in which watered and browsed individuals produced 1.8-3.6 times the number of spores compared to all other treatments (Table 3.3, Figure 3.3).

Spore populations that were isolated from trap soil samples belonged to five taxa of AMF, *Glomus intraradices, Paraglomus occulutum, Glomus sp., Scutellospora calospora,* and *Scuttellospora pellucida*. On average 84% of spores could be assigned to these five taxa. The remaining spores were unidentified species.

'Trap' isolated spores had higher species richness in browsed treatments than unbrowsed in both years (Table 3.3, Figure 3.3). Herbivory alone also induced a marginally significant increase in species diversity in 2008 (p=0.07) and a significant increase in 2009 (p= 0.004). There were no significant effects of water or interactions between herbivory and water for richness in either year (Table 3.3, Figure 3.3).

In contrast to the taxa identified in root samples (see TRFLP data above), species diversity of 'trap' soils increased in plants that received a water treatment compared to unaltered plants in both years (Table 3.3, Figure 3.3). There were significant interactions between browsing and water on species diversity in both years (Table 3.3, Figure 3.3). There was no significant difference in diversity between browsed and unbrowsed plants following a water treatment in either year.

In 'trap culture' soils, in which species were directly identified, browsing led to a significant decrease in the number of *P. occulutum* spores/30g of soil (with and without water additions in both years) and a significant increase in the number of spores/30g of soil for both

Scuttellospora species (with and without water additions in both years) (Table 3.4, Figure 3.4A, B). The number of *Glomus intraradices* spores significantly decreased under browsing in normal conditions of 2008 but were not significantly different in the drought conditions of 2009. However, in both years water alone significantly increased spores numbers (Table 3.4, Figure 3.4A). Water additions significantly increased spore production for *Glomus sp.* in 2008 but had no significant impact in the drought conditions of 2009 (Table 3.4A, Figure 3.4A). Water increased the spores of *S. pellucida* in both years (Table 3.4, Figure 3.4B). In 2008, the number of unidentified species was significantly increased by herbivory in 2008 and water in 2009 (Table 3.4, Figure 3.4B).

Discussion

Fungal Structural Allocation Patterns: Effects of Herbivory and Water

Overall, root colonization was significantly greater in 2009 than 2008, with higher percentages of hyphae, arbuscules, internal spores, vesicles, and DSE, independent of most treatment combinations (except for the watering treatments in which there were no significant differences in vesicles between 2008 and 2009). Of particular note, browsed plants had significantly higher percentages of AMF hyphae and arbuscules than unbrowsed plants under the drought conditions of 2009. These results are consistent with our previous findings that soil fungal communities were more parasitic on the compensatory response in drought conditions in 2009 than normal conditions of 2008 (Chapter 2), in that higher colonization of AMF may translate to a fitness decline of the host plant. We suspect that the drought conditions of 2009 and the added effects of herbivory lead to higher infection rates of mycorrhizal fungi seeking carbon in a community of plants diminished by drought. It could be argued that the increase in AMF hyphae and the number of arbuscules would facilitate the exchange of carbon, water, phosphorous

and other nutrients (Augé 2001, Augé 2004, Smith and Read 2008); however, under water-limited conditions, there is likely competition for carbon, particularly under regrowth conditions following herbivory (see e.g., Gehring and Whitham 2002). It has been shown that phosphorus uptake and transfer is lowered when photosynthate supplied to the fungi is reduced (Bücking and Shachar-Hill 2005), which would negatively impact plant fitness, as previously observed. Some arbuscular mycorrhizae are also known to be poor symbionts, providing little phosphorus while taking relatively high amounts of carbon, which could also lead to the observed reductions in fitness (Smith et al. 2004). Vesicles increased only in the drought of 2009 for browsed and unbrowsed plants relative to 2008. The increase in vesicles may represent a response to water-limited conditions in which vesicles store reserves necessary for building new cells under carbon limitation.

In addition, as noted above, drought conditions of 2009 increased the proportion of DSE colonizing roots over average precipitation in 2008. DSE lack a perifungal membrane for nutrient exchange, thus the % root colonized by DSE may not be associated with improved plant performance (Newhsam 2011). DSE has been proposed to be of benefit by protecting plants from soil pathogens (Newsham 2011). In this system, plants may not be challenged by pathogens to the extent that would gain a benefit from a DSE association, given that there were less than 2% colonization of non-AMF/DSE structures.

Water additions predominantly increased the proportion of arbuscules and internal spores (Figure 3.1A). The increase in the percentage of arbuscules formed was likely a response by fungi to an increase in photosynthate production. There was also a significant interaction between water and browsing for internal spores (Table 3.1A), with browsed plants significantly increasing spore production in both years following a water treatment, as well as a significant increase for

unbrowsed plants in 2008 following a water treatment. Water likely served as a trigger for spore production. Water additions also tended to improve the fitness of browsed plants and significantly enhanced the fitness of unbrowsed plants (Chapter 2). Nonetheless, water additions in 2009, didn't bring water availability to normal levels. A water addition was 34% below average rainfall and may explain why we didn't see reduced patterns of colonization on par with that observed in 2008.

Species Distribution Patterns: Effects of Herbivory and Water

Ungulate herbivory on *I. aggregata* also altered the composition of AMF within roots. The structure of AMF communities within roots showed that "species" diversity was significantly greater in browsed plants in 2008 and on average, higher in diversity in 2009 for browsed plants. There were no effects of water or water X browsing interactions on richness or diversity in either year. The increase in diversity may be a due to the root becoming a sink for carbon, produced from regrowth tissue following ungulate browsing. In addition, an increase in carbon may have allowed for greater number of AMF to colonize plant roots (Smith and Read 2008).

Ungulate herbivory on *I. aggregata* also altered the composition of AMF within 'trap' soils. In rhizosphere soils, species diversity and richness were significantly higher for browsed plants in 2009 with a marginally significant trend toward higher diversity and richness in 2008. Water additions significantly enhanced diversity in both 2008 and 2009. There were significant water X browsing interactions for diversity in 2008 and 2009 (see Figure 3.3). Spore abundance was also significantly higher in the rhizosphere soils of browsed plants. Herbivory has also been shown to increase spore counts by stimulating root growth (Frank et al. 2002, Gange 2007), as previously observed (Chapter 2). Water significantly enhanced spore production in the drought year of 2009 but not in 2008 (with normal levels of precipitation). Water alone had an effect in

rhizosphere soils but not in roots, enhancing overall spore production in soils in the drought year of 2009.

Herbivory and water impacted species diversity in both years. Both variables may create conditions that allow for a greater number of AMF species to sporulate in soils. In drought, species diversity of 'trap' soils and spore abundances in field soils were significantly higher in browsed plant rhizosphere soils. Although water contributed to an increase in diversity, when plants were browsed there was no significant differences in browsed plants with and without a water treatment.

Overall, root and rhizosphere soils show a similar result, most notably that ungulate herbivory enhanced, or tended to enhance, the richness and diversity of the AMF community. Of course, richness measures generally uncovered higher numbers of species in soils than in roots, given that the majority of species in soils were directly identified and those in roots were indirectly assessed using 18S rRNA sequences in which several species likely share identical band (TRF) sizes decreasing the number of species one can identify in roots. It is also interesting that water and water X browsing interactions affected diversity and spore abundance in soils but not in roots. This may be due to the direct availability of water to soil hyphae versus water available in roots, in which AMF compete for water resources with plants.

In 'trap' soils, in which species were directly identified via spores, browsing led to a decrease in the number of *P. occulutum* spores (with and without water additions in both years) and an increase in the number of spores for both *Scuttellospora* species (with and without water additions in both years). The number of *Glomus intraradices* and *Glomus sp.* spores decreased under browsing in the drought conditions of 2009. Water significantly increased spore production for most species under drought conditions. These patterns help to explain the fitness response of *I.*

aggregata to ungulate herbivory. Assuming that the species identified in the soil are also those found in the roots of *I. aggregata*, and are in the same proportions in the roots as those found in the soils (such correlations have been previously observed, e.g., see Bever 2002), the increase in *Scuttellospora* species in particular could also contribute to the fitness declines observed (Figure 6), along with the increase in structural allocation to AMF hyphae and arbuscules in browsed plants in 2009. *Scuttellospora calospora*, in particular, uses a large amount of photosynthate and has a poor capacity for translocating phosphorus (Jakobsen et al. 1992, Pearson and Jacobsen 1993). Given that diversity and richness were at their highest following ungulate herbivory, it would appear that *Scuttellospora calospora*, may represent a "super fungus" (Bennett and Bever 2007), negatively driving plant fitness. Of particular note, the abundance of the two *Scutellospora* species was exceedingly low and *Paraglomus occultum* and the two *Glomus* sp. predominated on unbrowsed plants in both years. Removal of these fungal species in the drought year had no significant effect on plant fitness but was parasitic under normal levels of precipitation, given that their removal significantly enhanced plant fitness. These results suggest that these fungal species become parasitic when carbon and water are less limiting.

Summary

Results here show that both water availability and herbivory had significant effects on levels of AMF colonization (hyphae, arbuscules, vesicles and spores), DSE, and AMF species distribution patterns. These changes could be linked back to results showing that fungal communities have a negative effect on compensatory growth (Chapter 2). Further research is needed to experimentally assess the impacts of each AMF species independently and in all combinations and under varying environmental conditions on the regrowth response of *Ipomopsis aggregata* following ungulate browsing.

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Tables and Figures

Table 3.1. Analysis of variance (ANOVA) results for % fungal colonization (hyphae, arbuscules, vesicles, internal AMF spores (i.spore), non-AMF/DSE fungi (non), and dark septate endophytic fungi (DSE) of *Ipomopsis aggregata* plant roots, with and without ungulate herbivory, and water treatments in 2008 and 2009.

			Hyphae		arbuscules		Vesicles		i. spores		non		DSE	
		df	F	P	F	P	F	P	F	P	F	P	F	P
2008	Herbivory	1	1.2	**0.05**	0.3	0.58	1.45	0.23	22.54	**0.06**	0.01	0.92	0.34	0.56
	Water	1	0.61	0.44	5.96	**0.002**	0.84	0.36	24.84	**<0.001**	0.55	0.45	0.93	0.34
	H x W	1	0.93	0.34	0.02	0.88	0.04	0.84	10.96	**0.001**	0.36	0.55	0.05	0.82
	Error df	152												
2009	Herbivory	1	202.5	**0.03**	39.99	**<0.001**	0.24	**0.04**	16.16	**0.05**	1.97	0.17	3.97	0.16
	Water	1	13.7	**0.05**	7.56	**0.003**	18.55	**<0.001**	41	**0.02**	3.01	0.09	3.6	0.17
	H x W	1	3.02	0.09	0.2	0.66	13.73	**<0.001**	43.29	**0.05**	1.21	0.28	2.56	0.12
	Error df	44												

Notes: F-values (F) with significance levels (P) and degrees of freedom (df) are given.

Table 3.2. Negative Binomial Model results for spore number and species richness, and ANOVA results for species diversity for number of arbuscular mycorrhizal fungal spores collected from *Ipomopsis aggregata* rhizosphere soils and species richness and diversity of trap cultures inoculated with soils and root fragments with and without ungulate herbivory and a water addition in 2008 and 2009.

			spore		richness		diversity	
		df	z	P	z	P	F	P
2008	Herbivory	1	-4.47	**<0.001**	-1.77	0.08	2.91	0.07
	Water	1	0.05	0.96	0	1	53.13	**<0.001**
	H x W	1	1.38	0.17	1.18	0.24	14.02	**<0.001**
	Error df	36						
2009	Herbivory	1	-4.38	**<0.001**	-1.19	**<0.001**	10.01	**<0.01**
	Water	1	3.96	**<0.001**	0	**1**	57.9	**<0.001**
	H x W	1	-1.72	0.08	0.63	0.53	9.3	**<0.01**
	Error df	36						

Notes: z-values (z) for spore no. and richness and F-values (F) for diversity with significance levels (P) and degrees of freedom (df) are given.

Table 3.3. Analysis of variance (ANOVA) results for dominant arbuscular mycorrhizal fungal (AMF) species collected from trap cultures inoculated with rhizosphere soils of *Ipomopsis aggregata* plants and root fragments with and without ungulate herbivory, and water treatments in 2008 and 2009. The AMF species were *Paraglomus occulutum* (*P. o.*), *Glomus intraradice*s (*G. i.*), an unidentified *Glomu*s sp. (*G*. sp.), *Scuttellospora callospora* (*S. c.*), *Scuttellospora pellucida* (*S. p.*), and all remaining unidentified species (other sp).

			P.o.		*G. i.*		*G.* sp.		*S. c.*		*S. p.*		other sp.	
		df	SS	p	SS	*p*	SS	*P*	SS	*P*	SS	*p*	SS	*p*
2008	Herbivory	1	5382.4	**<0.001**	1562.5	**<0.01**	176.4	0.12	2088.02	**<0.001**	6175.2	**<0.001**	7144	**<0.001**
	Water	1	193.6	0.29	184.9	0.28	2044.9	**<0.001**	34.23	0.52	1071.2	**<0.01**	533.2	0.1
	H X W	1	193.6	0.29	1254.4	**<0.01**	2.5	0.85	140.63	0.2	99.2	0.33	279.8	0.22
	Error df	36	6014		5500.2		2564.6		2933.1		3670.3		181.5	
2009	Herbivory	1	3666.4	**<0.001**	628.5	0.17	358	0.15	2822.9	**<0.001**	5266.9	**<0.001**	1700.4	0.01
	Water	1	365	0.18	4083.2	**0.001**	699.6	**0.05**	586.3	**0.05**	3144.8	**<0.001**	7606.4	**<0.001**
	H X W	1	100.8	0.48	465.4	0.24	114.9	0.41	302.4	0.15	447.6	**0.05**	404.3	0.21
	Error df	29	5606.8		9187.6		4745.1		3937.4		3078.7		246.8	

Notes: Sum of Squares (SS) (note: need to put in z-values, instead) with significance levels (P) and degrees of freedom (df) are given.

Figure 3.1. Measurements of % of arbuscular mycorrhizal fungal colonization (A) (+/- S.E.) (hyphae, arbuscules, vesicles, and internal spores) and non-arbuscular mycorrhizal fungi colonization (B) (+/- S. E.) (dark septate endophytic fungi (DSE) and all other non-AMF fungal structures (non)) in *Ipomopsis aggregata* roots with and without herbivory and water treatments in 2008 and 2009.

A

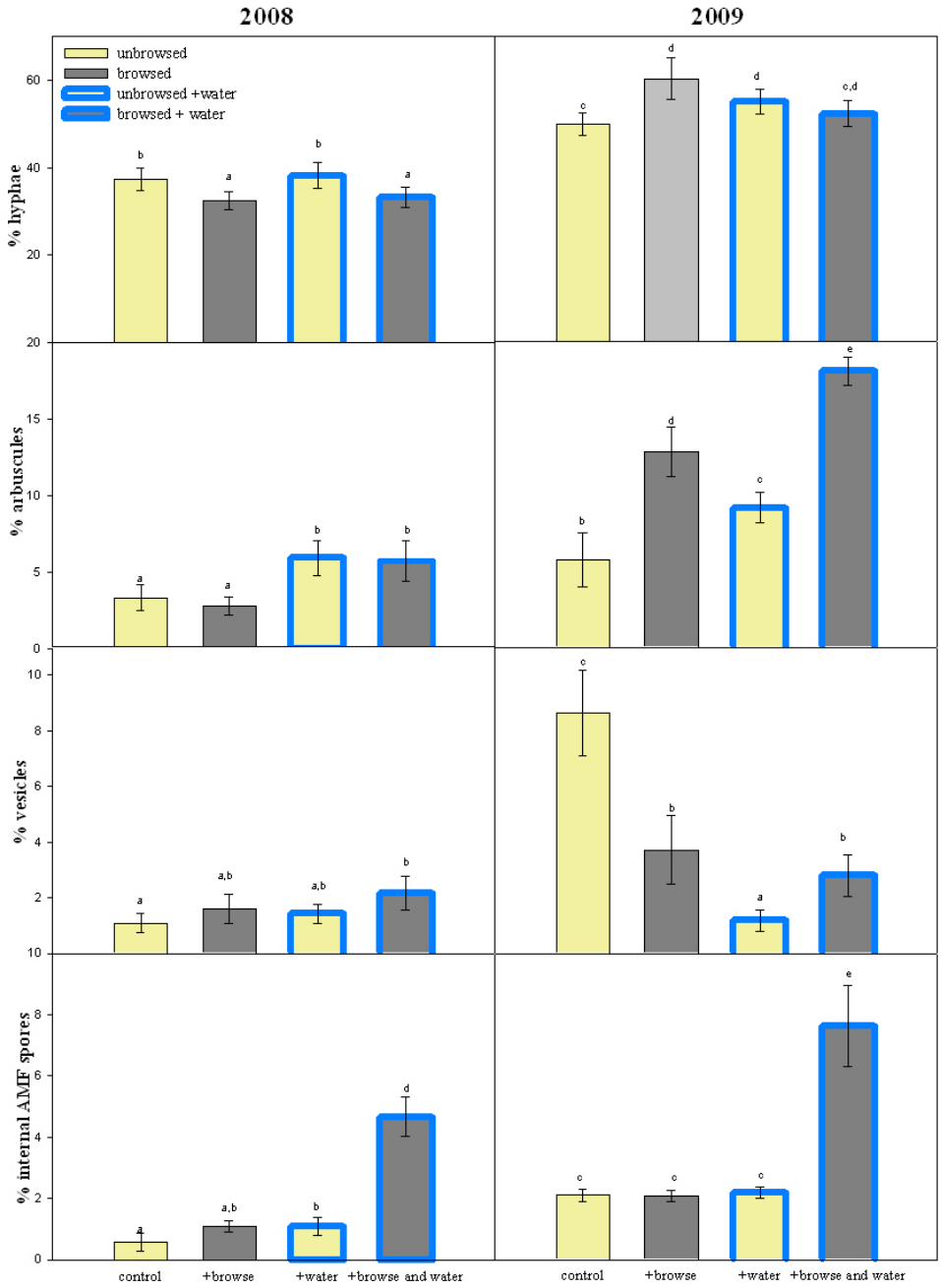


Figure 3.1 (cont.)

B

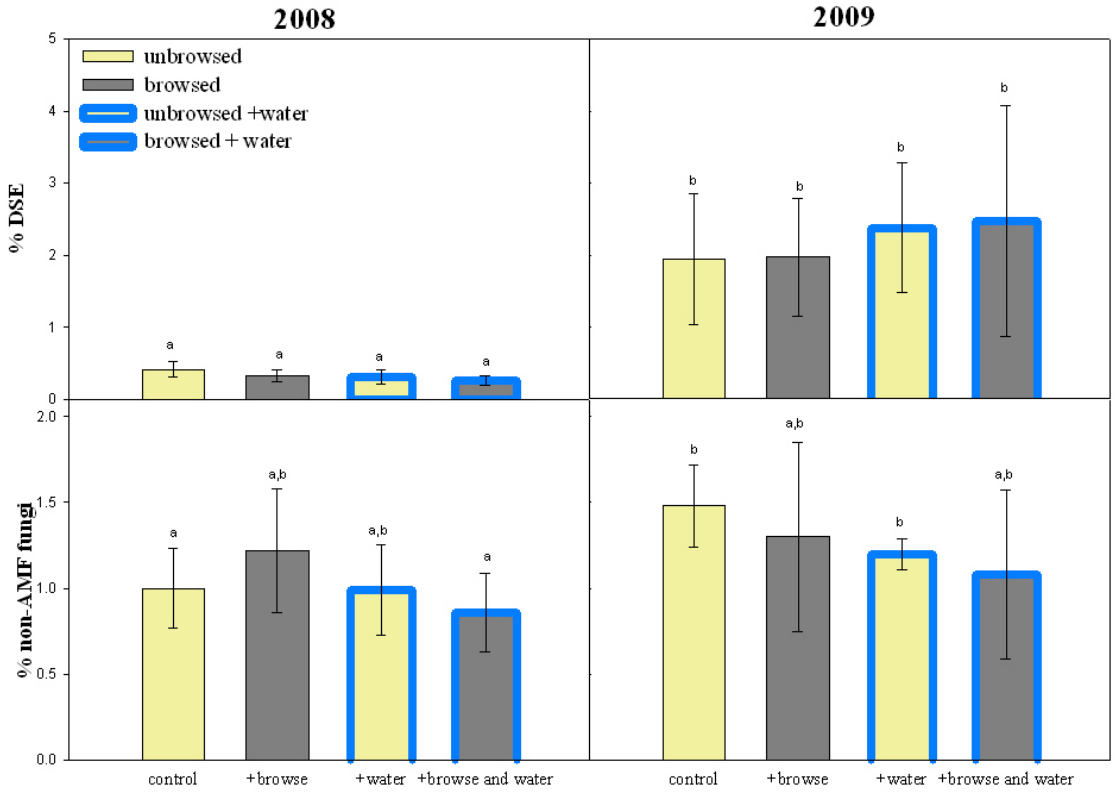


Figure 3.2. Arbuscular mycorrhizal fungal total fluorescence (+/- S. E.) and species richness and diversity employing TRFLP (Terminal Restriction Fragment Polymorphism) data with and without ungulate herbivory and a water treatment in 2008 and 2009.

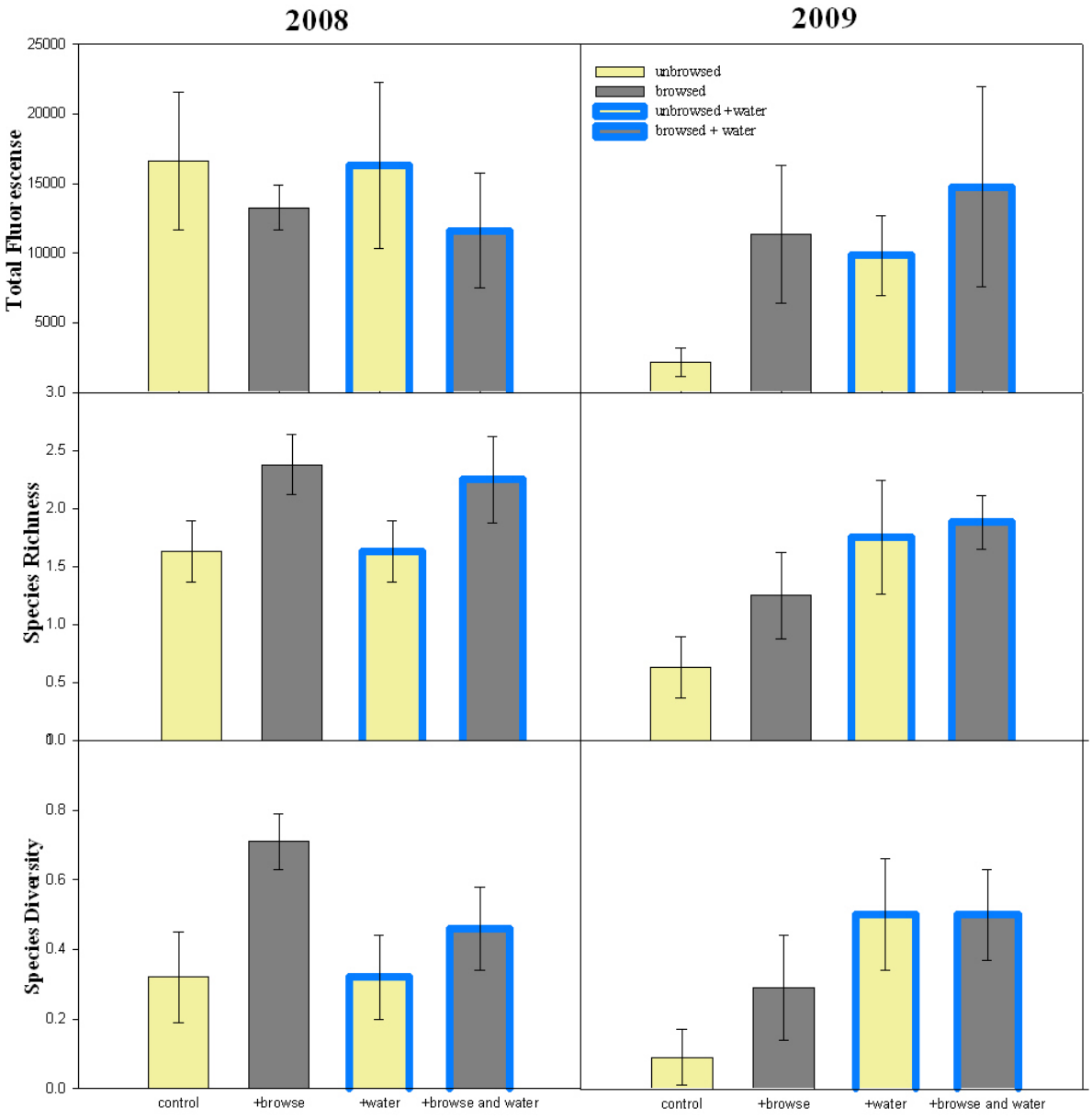


Figure 3.3. Average arbuscular mycorrhizal (AMF) spore number (+/- S. E.) collected from *Ipomopsis aggregata* rhizosphere soils, and AMF species and diversity, both collected from trap cultures inoculated with *I. aggregata* rhizosphere soils in 2008 and 2009. All variables are with and without herbivory and a water addition.

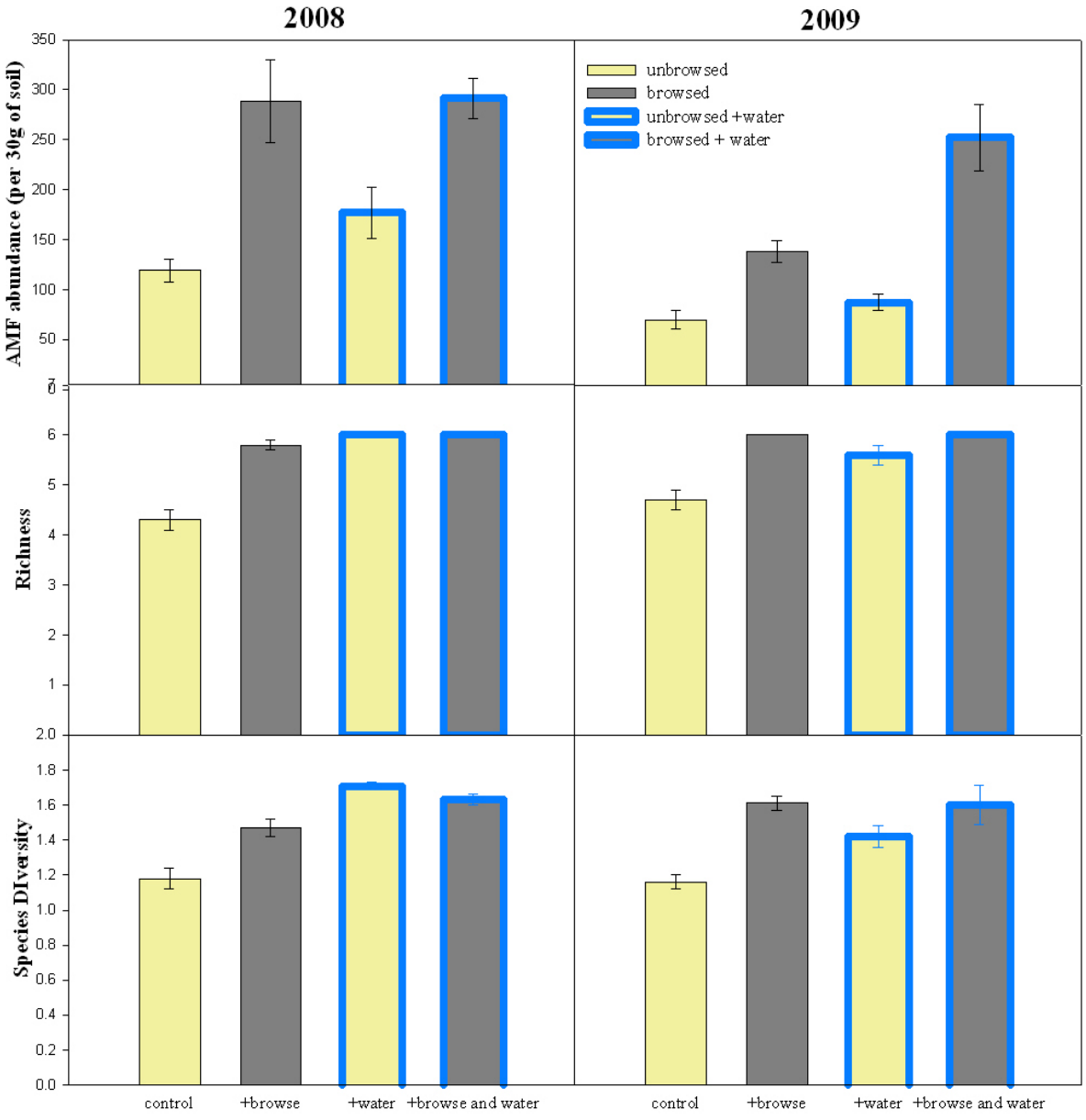


Figure 3.4. Average number (+/- S. E.) of smaller-spored (A) arbuscular mycorrhizal fungal species (*Paraglomus occulutum*, *Glomus intraradices*, *Glomus* sp.) and larger-spored arbuscular mycorrhizal fungal species (B) (*Scuttellospora calospora*, *Scuttellospora pellucida* and an unidentified bin of AMF) from trap cultures inoculated with *Ipomopsis aggregata* rhizosphere soils with and without ungulate herbivory and water treatments in 2008 and 2009.

A.

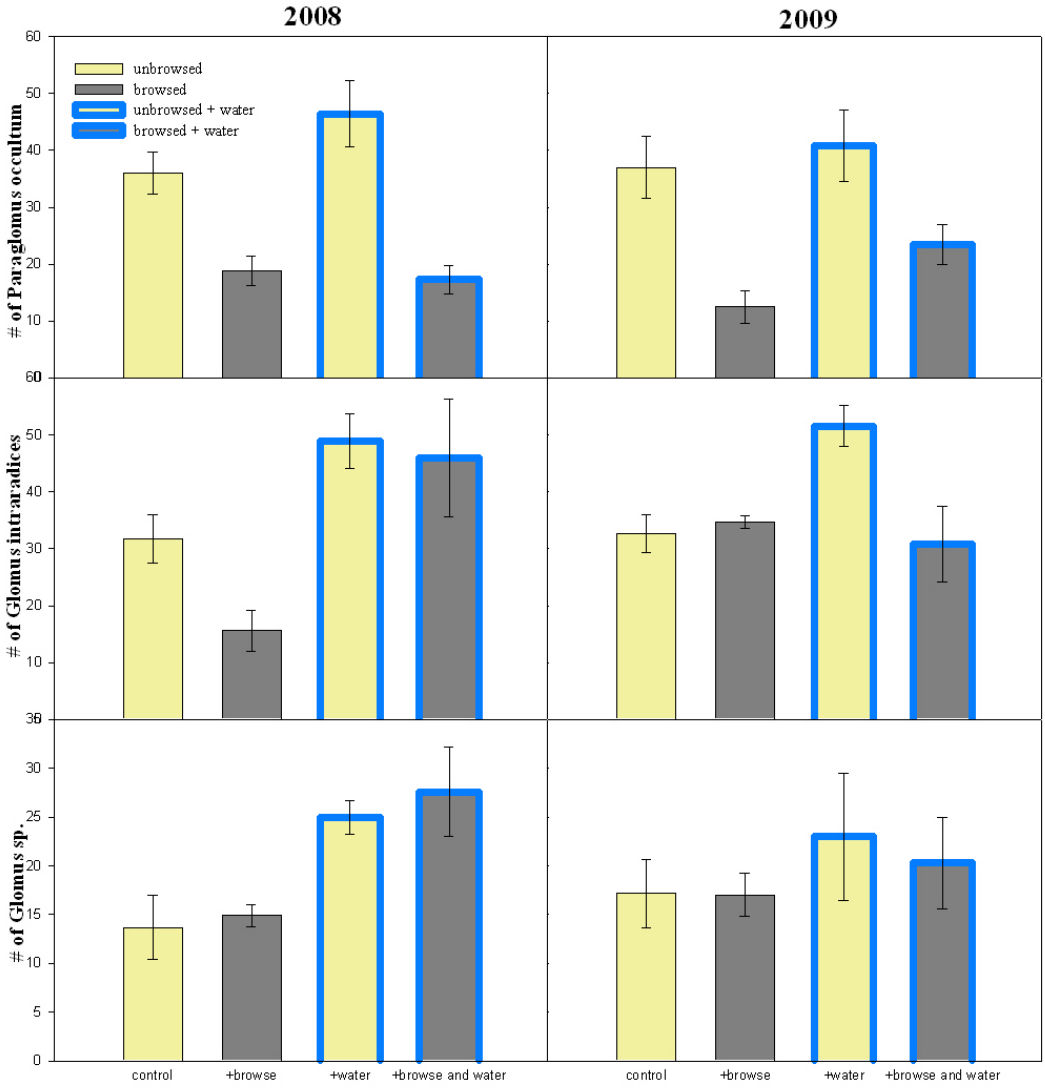
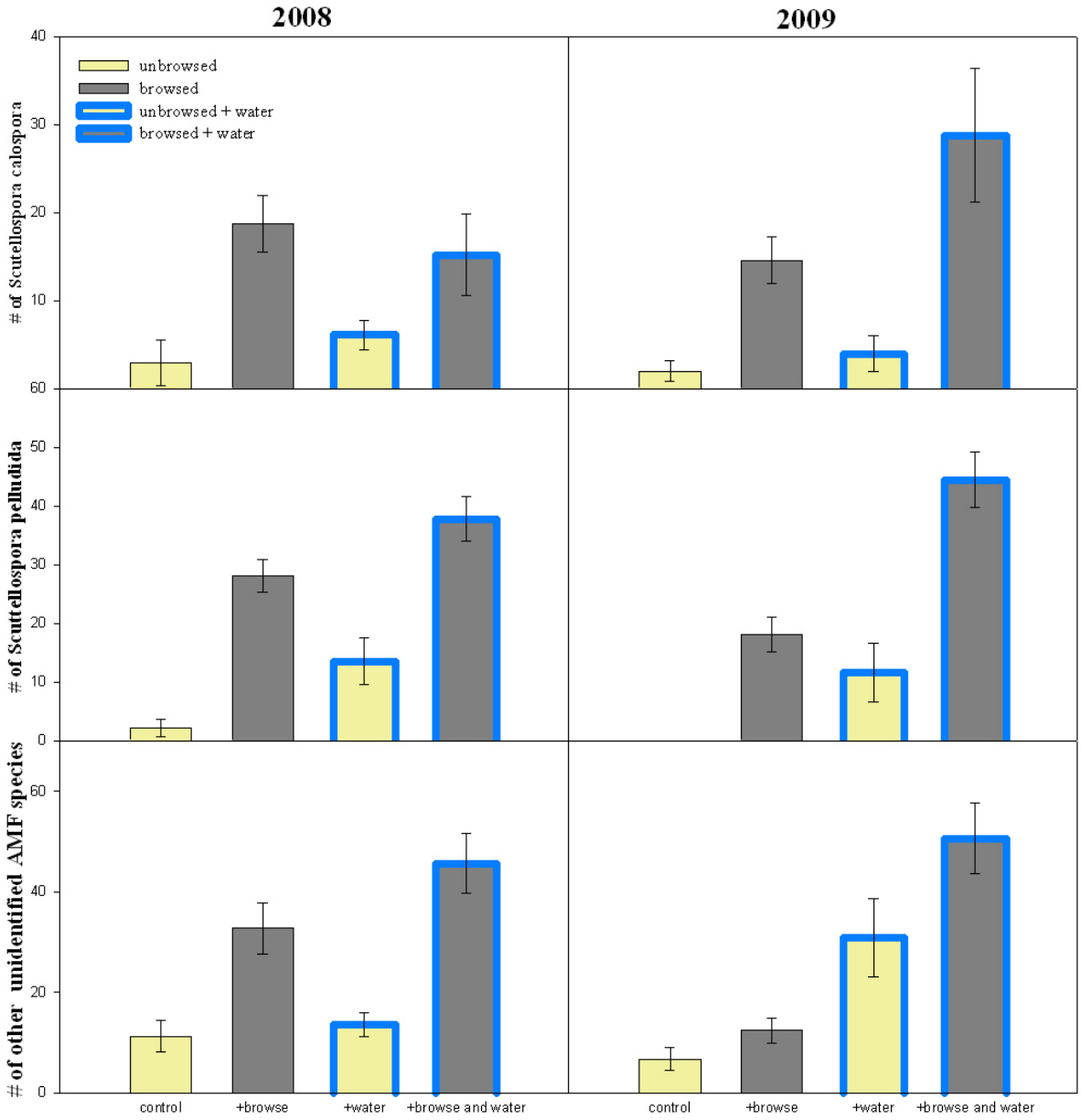


Figure 3.4 (cont.)

B



CHAPTER 4: ROOT FUNGAL COMMUNITIES INTERACT WITH NUTRIENT AVAILABILITY TO DETERMINE COMPENSATORY RESPONSE OF *IPOMOPSIS AGGREGATA*

Abstract

Here, we extend our studies on the interactive effects of herbivory and soil fungal communities on the compensatory response of *Ipomopsis aggregata* by incorporating a series of P treatments to assess the interactive effects of P on plant compensation and arbuscular mycorrhizal colonization in a multi-factorial design. Results show that soil nutrient levels and the soil inhabiting fungal community interact with herbivory to determine the growth and compensatory response of scarlet gilia. Specifically, the compensatory response is limited by P availability, with nutrient availability being more important for browsed plants than for unbrowsed plants. Phosphorus is also shown to decrease mycorrhizal allocation to hyphae, arbuscules and internal spores within plant roots. Whereas plants equally compensated under low nutrient conditions, they overcompensated under the highest level of P, due to an increase in the fitness of browsed plants. Furthermore, the removal of soil fungi with a fungicide treatment resulted in overcompensation under the lowest and highest nutrient conditions, due primarily, to an increase in the fitness of browsed plants. These results support the findings of others that under high P conditions plants shift to soil P when plants are able to obtain nutrients through their own root systems, decreasing fungal colonization and enhancing plant fitness following herbivory.

Introduction

Herbivory, soil fungal communities, and soil nutrient availability play key roles in shaping plant performance (Crawley 1997, Smith and Read 2008, Ruotsalainen and Eskelinen 2011). The consequences of herbivory are often negative due to a loss of photosynthetic biomass.

However, in conditions with high resources, the effects of herbivory may be neutral or even beneficial due to regrowth strategies that can supplant or exceed biomass lost (Piippo et al. 2011). It has been assumed that plants growing in high resource conditions are best able to compensate for herbivory (Bryant et al. 1983, Coley et al. 1985, Maschinski and Whitham 1989). However, just the opposite has been found for dicotyledonous plants exhibiting patterns of overcompensation (increased seed yield following herbivory) with most occurring in resource-poor conditions (Hawkes and Sullivan 2001, Wise and Abrahamson 2007). These studies, however, have disregarded the potential effects of belowground interactions root inhabiting fungi that could provide the necessary nutrients. Under nutrient-limited conditions, associations with AMF could provide nutrients (particularly phosphorus) for the host plant in exchange for photosynthates (Smith and Read 2008). Of course, exchange among plants and soil fungi may be altered by herbivory wherein the removal of a significant amount of biomass will reduce the carbon producing capacity and nutrient needs of the host plant.

Resource availability has also been shown to mediate plant-AMF relationships (Johnson et al. 1997, Koide 1991). In conditions of high nutrient availability, AMF colonization may be reduced (Gehring and Whitham 2002). As nutrients increase in soils, plants may reduce costs by obtaining nutrients through their own root systems rather than through a fungal association (Tuomi et al. 2001, Thrall et al. 2006). Alternatively, high nutrient availability could select for fungal taxa that are parasitic, increasing the level of colonization (see Chapter 3) beyond the optimal for the host plant.

A long-term study of the monocarpic biennial, scarlet gilia, *Ipomopsis aggregata*, has shown that ungulate herbivory can lead to overcompensation (enhanced fitness) in northern Arizona (e.g., Paige and Whitham 1987, Paige 1992, 1999). In this system, soil fungal

communities had negative effects on compensatory growth. Their removal led to an overall increase in fitness for browsed plants (and unbrowsed plants when water was not a limiting factor; Chapter 2). The parasitic nature of the relationship could potentially stem from fungi withholding nutrients (particularly phosphorus) from the plant when the fungus is connected to a host that is of low benefit (Smith and Read 2008, Hammer et al. 2011). A previous study also indicated that browsing led to an increase in *Scuttellospora sp* (Gigasporaceae family), that have larger spores than other AMF species. *Scuttellospora* may not be the most beneficial species in that they take relatively large amounts of carbon while translocating little phosphorus to the plant (Pearson and Jakobsen 1992, Pearson and Jakobsen 1993, Smith et al. 2000, Kiers et al. 2011). These results may explain the increase in fitness observed in browsed plants following fungal removal (Chapter 2).

Examinations of the interactive effects of herbivory and soil fungal associations are expanded upon by integrating nutrient availability as a potential predictor of a plant's ability to compensate. The following three questions are addressed: 1). Is the compensatory response of *I. aggregata* limited by P in native soil? Is there a differential effect of P on unbrowsed versus browsed individuals? 2). Do increasing levels of P inhibit or enhance AMF colonization? 3). Do increasing levels of P alter previously observed parasitic effects of fungal communities on compensation of *I. aggregata*?

Study Site and Organisms

Experiments were conducted in Coconino County, 20 miles northwest of Flagstaff, Arizona, USA (altitude approximately 2500 meters). This population averaged 30,000 flowering individuals of scarlet gilia, *Ipomopsis aggregata*, surrounded by ponderosa pine (*Pinus ponderosa*) and aspen (*Populus tremuloides*). Scarlet gilia is a biennial, monocarpic herbaceous

plant that is abundant in montane meadows of the western United States (USDA plant database). They spend their first year as a vegetative rosette and in the second year a single paniculate inflorescence bolts in early spring and flowers by mid-July to early August. Mule deer (*Odocoileus hemionus*) and elk (*Cervus canadensis*) browsed 91% of all bolting scarlet gilia plants in 2009, the year of this study (Allsup, personal observation).

The fungal community in scarlet gilia roots at this site consists mainly of arbuscular mycorrhizal fungi (AMF) characterized by arbusucles (finely-branched tree-like structures) and coenocytic hyphae (lacking cross-walls), on average they represent approximately 55-95% of total colonization (Chapter 3). In addition, ascomycetes, dark septate root endophytes (DSE) characterized by dark septate hyphae and sclerotia comprise 2-4% total colonization. Less than 2% of total colonization is non-AMF/DSE root inhabiting fungi, such as oospores.

Methods

Experimental Design

A factorial design was conducted in 2009 (a moderate drought year) to determine the impacts of ungulate herbivory, the soil fungal community, and a P gradient on the compensatory response of scarlet gilia. In May, a 100*50 meter section of the Arizona field site was used to designate a baseline that was segmented into 20 transects, each 10 meters apart, to ensure a minimum mycorrhizal network between plants. Each treatment was represented within each transect. In this experiment, plants were unbrowsed or naturally browsed (see treatments below). Previous studies (Paige and Whitham 1987, Paige 1992a, 1994, 1999) have failed to show significant differences in fitness between naturally browsed and experimentally clipped plants (chosen from the pool of uneaten individuals following natural herbivory). These results argue that plant selectivity by herbivores had no effect on scarlet gilias' ability to compensate, or

alternatively, that herbivores were not selective (Anderson and Paige 2003). Unbrowsed individuals represent less than 15-20% of the population, and were harder to find so, on a few occasions we had to go outside the range of assigned placement. Initial stem diameter, used as a size covariate, was taken and plants were assigned to each combination of treatments: unbrowsed (U) or browsed (B), fungicide (F) or no fungicide (N), and no P addition (P_0) or one of three levels of nutrient addition (P_1, P_2, and P_3).

Fungicide was administered throughout the growing season every two weeks for a total of five treatments to the base of each plant. Each total treatment consisted of 2.52 grams of the fungicide captan in 1420 mL water (Southern Agriculture Insecticides, Palmetto, Florida). A single treatment consisted of 0.5 grams of the fungicide captan in 0.28 L of water. Captan (cis-N-trichloromethyl thio-4-cyclohexane-1, 2-di- carboximide) reduces fungal colonization by halting cellular respiration of fungi (Kough et al. 1987). The remaining non-fungicidal controls received an equivalent amount of water to mimic the liquid that fungicide treated plants were given. A fungicide treatment reduces total fungal colonization of plant roots by approximately half (Chapter 2).

The phosphorus treatment was comprised of a powdered Super Phosphate Fertilizer 0-46-0 mixed with water and added to the base of the plant once in May. The three P addition treatments were determined by 1.5X, 2X, and 2.5X the mean (ppm) of native soil equivalent to 31.05 ppm M3P or 27.45 kg/acre. P treatments were: P_1 = 41.18 kg/acre, P_2 = 54.9 kg/acre, and P_3 = 68.63kg acre. P additions included: P_1 = 2.27g, P_2 = 3.41g, and P_3 = 4.54g in 1 cup of water per plant.

Plant/root assessment

At the end of the growing season in early September, an average of 16 plants (range 13-17) were collected for each treatment. Above and belowground biomass was separated, weighed. Roots were stored in a -40°C degree freezer. Plant fitness was measured as the number of fruits, seed mass, and aboveground and belowground biomass for each plant/treatment combination. Seed mass was taken from a subsample of the experiment, 10 fruit from 10 plants per treatment.

To determine changes in fungal "fitness" due to the interactive effects of herbivory and nutrients, root colonization and structural allocation (hyphae, arbuscules, vesicles and spores) were determined using direct blue staining method (INVAM). Approximately 15 mg of roots were removed from live plants, washed, and placed in plastic cassettes. Plants were cleared with a pre-boiled 10% potassium hydroxide (Gardner 1975). After 24 hours, roots were rinsed and bleached with hydrogen peroxide for an hour, rinsed, and then acidified with 2% hydrochloric acid for 6 hours. The roots were stained with a pre-boiled Direct Blue solution (glycerol:lactic acid:water) for 7 minutes. Following the stain procedure, roots were soaked in water to destain. Colonization was determined using the magnified grid line intersection methods (McGonigle 1990) under 1000X magnification. Results were reported as percentage per root length with AMF hyphae, AMF arbuscules, AMF vesicles, AMF spores, DSE, and non-AMF fungi.Not all fungi will uptake the stain. For example, DSE are not as able to uptake chitin-adhering dyes (Barrow and Aaltonen 2001, Mandyam and Jumponen 2005).

Statistical Analysis

A GLM with a negative binomial distribution was employed for count data, fruit number, and separately for plants administered a fungicide treatment and non-fungicide-treated plants. Stem diameter, measured at the start of experiment, was used as a covariate to control for initial

plant size. The dependent variables were herbivory (unbrowsed and browsed), and all four levels of P (natural P_0, P_1, P_2, and P_3). Variance was controlled for browsing treatment. For continuous variables (above-, below-ground biomass, and seed mass), analysis of variance (ANOVA)s was performed for continuous data using the same dependent variables, as above. Additionally, an ANOVAs wer performed on the proportion of fungal structures, including, hyphae, arbuscules, vesicles, internal spores, and non-AM fungi. The dependent variables for fungal "fitness" were herbivory (browsed or unbrowsed), and all four levels of P (P_0, P_1, P_2, and P_3). Post hoc ANOVAs were performed for treatment effects.

Results

The compensatory response of scarlet gilia was limited by P availability in native soils, in terms of fruit production (Figure 4.1). There was a significant interactive term for herbivory X P treatment for fruit number and aboveground biomass (Table 4.1). In native soils and the first two P additions, plants equally compensated (fruit number of browsed plants equaled those of unbrowsed plants (P_0- $P=0.43$, P_1-$P=0.26$, P_2-$P=0.06$) but, at the highest level of P additions (P_3) plants overcompensated (P_3-$P=0.02$) (Figure 4.1). Above- and below-ground biomass also increased with higher levels of P; browsed plants were significantly bigger with P additions when compared to unbrowsed individuals (above - P_1-$P=0.006$, P_2-$P<0.001$, P_3-$P=0.008$, Figure 4.1, below - P_2-$P<0.01$ P_3-$P=0.02$, Figure 4.1). A P addition had no impact in terms of seed mass (Table 4.1, Figure 4.1).

P also affected unbrowsed and browsed plants differently (Table 4.1, Figure 4.1). Browsed plants with P additions (browsed individuals P_0 vs. P_1, P_2, and P_3) significantly increased the number of fruit produced ($P=0.03$), whereas unbrowsed individuals with P additions showed no significant differences in fruit production (Figure 4.1, $P=0.34$). There were

no significant differences for above- or below-ground biomass or seed mass for browsed plants (Table 4.1).

As P additions increased, AMF colonization generally decreased (Figure 4.2). Specifically, hyphae and internal AMF spores of both unbrowsed and browsed individuals decreased (Table 4.2, Figure 4.2). Additionally, browsed individuals decreased % arbuscules and vesicles, whereas unbrowsed plant roots increased % arbuscules and vesicles (with the exception of P_2) with P additions ($P_{arbuscules}<0.0001$, $P_{vesicles}= 0.03$, Figure 4.2).

P availability alters the impact of AMF on the compensatory response. With normal AMF association, as stated above, a P addition increased the compensatory response (Figure 4.1). AMF reduction with a fungicide treatment (Figure 4.1) resulted in an increase in the compensatory response ($P_{fung}<0.01$), from equal compensation to overcompensation, for fruit number in P_0 and P_1 treatments and a significant increase in browsed individuals in the highest phosphorus treatment, P_3 (, $P<0.001$ Figure 4.1). At an intermediate level (P2), the reduction of AMF did not improve compensation (P=0.63, Figure 4.1). The highest fitness value was observed in browsed individuals with a fungicide treatment in the highest P treatment, in that they had 1.6X or greater fruit compared to all other treatments (Figure 4.1). Aboveground biomass for browsed individuals was significantly greater for all P treatment combinations following a fungicide treatment ($P<0.001$, Figure 4.1). The belowground biomass of plants with a fungicide treatment decreased with P additions (P=0.05, Figures 4.1) and had no impact on seed mass (Table 4.1, Figure 4.1).

Discussion

Results of this study demonstrate that soil nutrient levels and the soil fungi community interact to determine the growth and compensatory response of scarlet gilia following herbivory.

The main finding extends the results of our previous studies on the interactive effects of herbivory, water availability and soil fungi on fitness compensation in *I. aggregata* (Chapters 2 and 3) in that 1) the compensatory response is limited by P availability, 2) nutrient availability is more important for browsed plants than unbrowsed plants, 3) an increase in P decreased mycorrhizal allocation to hyphae, arbuscules and internal spores within plant roots, 4) plants equally compensated under low nutrient conditions and overcompensated under the highest level of P, due to an increase in the fitness of browsed plants, and 5) the effects of fungicide was to allow overcompensation even at low P, but it had minimal effects on compensation at high P levels.

The compensatory response is, at least in part, dependent on P availability. Browsed plants were limited by P, whereas, unbrowsed individuals were not, given that unbrowsed plants gained no clear fitness benefit from P additions at any level. Thus, the compensatory response only occurred at high P additions. These results are consistent with the compensatory continuum hypothesis in which the compensatory response following herbivory is predicted to be greatest in high resource or less stressful conditions (Maschinski and Whitham 1989, Whitham et al. 1991). The significant increase in compensation by browsed plants following a high P treatment is likely due to the greater demand by these plants for nutrients and water following herbivory. The newly juvenalized regrowth tissues and enhanced photosynthetic rates require greater nutrient inputs, particularly limiting nutrients such as P. The increase in root biomass and spread in both the tap and fibrous portions of the root system may secondarily facilitate the accumulation and transport of nutrients and water contributing to enhance aboveground biomass and fruit production, particularly at higher P concentrations.

In addition, under high levels of P the percent colonization of hyphae, arbuscules and internal spores was significantly reduced, likely facilitating the observed increase in fitness in browsed plants by decreasing the competitive effects of the fungi (see Chapter2). Interestingly, unbrowsed plants increased arbuscule production following P treatments but showed no clear change in fitness across treatments. Nonetheless, hyphae in unbrowsed plant roots significantly decreased with increasing P treatments. Thus, arbuscular colonization increases may reflect the maintenance of the status quo in terms of fitness in unbrowsed plants. The differential impacts of P on unbrowsed and browsed plants could be related to shifts in mycorrhizal species composition (Chapter 3). Gigasporacea, the solely colonize browsed plants as opposed to unbrowsed plants (Chapter 3) tend to have lower rates of colonization than Glomeracea (greater abundance in unbrowsed individuals).

Following the removal of fungi with a fungicide treatment altered the compensatory response from equal compensation to overcompensation under natural levels of P by releasing the competitive effects of the fungal community. A 2.5-fold increase in the level of P, along with the removal of fungi, significantly enhanced the compensatory response of browsed plants approximately 2.0-fold over browsed plants with the same level of P but with fungi present (see Figure 4.1). Plants with soil fungi showed a joint response of compensation and colonization to increasing phosphorous. But the fact that compensation increased across P levels in the fungicide treatments suggests a causal role of AMF colonization (i.e. lower fungal colonization equates higher compensation). This is important because we have now manipulated AMF colonization in two ways, through fungicide and through fertilization (P additions). Both methods have artifacts, but importantly, they have different artifacts.

In addition, under high levels of P the percent colonization of hyphae, arbuscules and internal spores was significantly reduced by increasing levels of P, likely facilitating the observed increase in fitness in browsed plants by decreasing the competitive effects of the fungi. Interestingly, unbrowsed plants increased arbuscule production following P treatments but showed no clear change in fitness across treatments. Nonetheless, hyphae in unbrowsed plant roots significantly decreased with increasing P treatments. Thus, arbuscule increases may reflect the maintenance of the status quo in terms of fitness in unbrowsed plants.

Kiers et al. (2011) predicted that control in plant-AMF interactions would be bidirectional and equally beneficial to both partners. In this study, the decrease in AMF following increasingly higher P additions in browsed individuals would suggest that the plant has primary control over the AMF-plant relationship, reducing the AMF when P is abundant enough that the plant no longer needs to depend upon the AMF for supplying P. However, this interpretation is confounded when we consider that under drought conditions and natural levels of P, fungal colonization increases and plant fitness is significantly reduced in browsed plants (i.e., soil fungi are parasitic given that plant fitness is significantly enhanced when fungi are reduced). It would seem in this case that fungi dictate the relationship. On the contrary, if plants control colonization, through browsing events and soil nutrient condition, it could be suggested that plants are in control, at least partially. Overall, who dictates the relationship is likely condition dependent – certainly the strategies taken by plants and soil fungi are not mutually beneficial and for scarlet gilia predominantly detrimental in terms of maximizing fitness.

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Tables and figures

Table 4.1. Negative Binomial Model results for fruit number and analysis of covariance (ANCOVA) results for aboveground and belowground biomass and seed mass of *Ipomopsis aggregata* plants, with and without ungulate herbivory, and Phosphorus treatments in 2009.

	fruit			Above			below			seed		
No fungicide	df	z	P	df	F	P	Df	F	P	Df	F	P
Herbivory	1	-2.43	**0.02**	1	1.68	0.1	1	0.41	0.6	1	0.53	0.6
P	3	0.92	**0.04**	3	0.17	0.87	3	0.19	0.85	3	0.84	0.41
Stem	1	5.03	**<0.001**	1	5.34	**<0.001**	1	2.59	**0.02**	1	0.41	0.68
H X P	3	-0.06	**0.05**	3	1.31	**0.02**	3	0.69	0.49	3	0.46	0.65
Error	123			123			113			70		
Fungicide												
Herbivory	1	-4.74	**<0.001**	1	4.56	**<0.001**	1	2.46	**0.02**	1	0.69	0.49
P	3	0.87	0.39	3	0.3	0.77	3	2.05	**0.05**	3	0.61	0.55
Stem	1	4.95	**<0.001**	1	5.86	**<0.001**	1	3.36	**0.001**	1	1.38	0.17
H X P	3	0.99	0.32	3	0.35	0.73	3	0.94	0.35	3	0.93	0.36
Error	123			123			118			55		

Notes: Z-values (z) and F-values (F) with significance levels (P) and degrees of freedom (df) are given.

Table 4.2. Analysis of Variance (ANOVA) results for % arbuscular mycorrhizal fungal (AMF) colonization of hyphae, arbuscules, vesicles, internal spores, and non-AMF structures in *Ipomopsis aggregata* roots with and without herbivory and Phosphorus treatments in and 2009.

		hyphae		arbuscules		vesicles		spores		non-AMF	
	Df	F	P	F	P	F	P	F	P	F	P
Herbivory	1	0.52	0.48	10.7	**0.002**	0.75	0.39	0.49	0.49	2.08	0.16
P	1	33.39	**<0.001**	4.45	**0.04**	0.5	0.48	8.78	**0.004**	0.01	0.91
H X P	1	<0.01	0.99	17.55	**<0.001**	5.26	**0.03**	0.46	0.5	6.43	**0.01**
Error df	58	6679.7		449.6		199.68	3.44	172.39		36.67	

Notes: F-values (F) with significance levels (P) and degrees of freedom (df) are given.

Figure 4.1. Average fruit number, aboveground and belowground biomass, and seed mass (+/- S.E.) of *Ipomopsis aggregata* plants with and without ungulate herbivory, a fungicide treatment, with native soils (P_0) and three P additions.

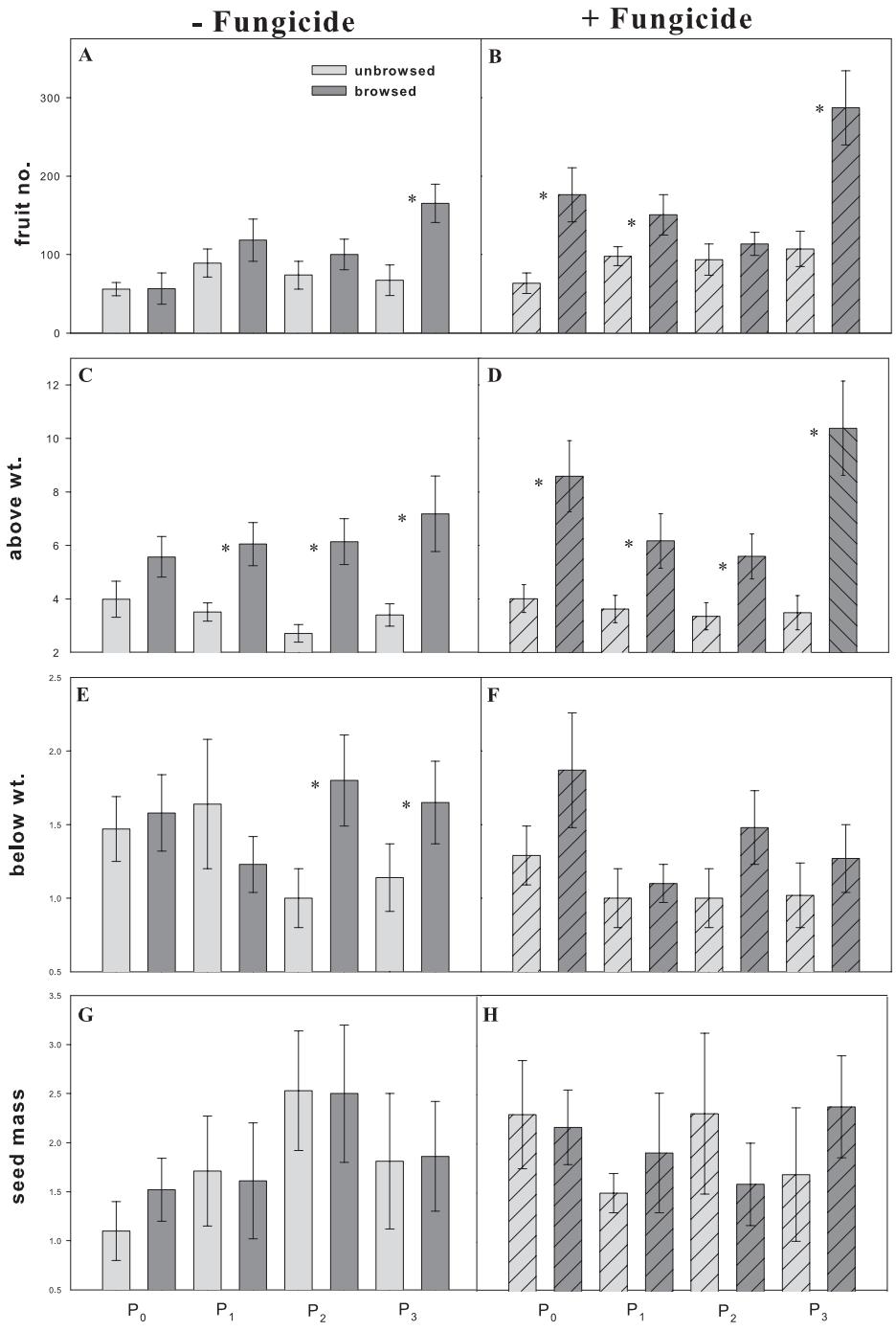

Figure 4.2. Measurements of arbuscular mycorrhizal fungal (AMF) % colonization (+/- S. E.) (hyphae, arbuscules, vesicles, and internal spores) of *Ipomopsis aggregata* roots with and without ungulate herbivory and with native soils (P0) and three P additions.